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New Insight Into the Role of the Cdc34 Ubiquitin-Conjugating Enzyme in Cell Cycle Regulation via Ace2 and Sic1

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ABSTRACT

The Cdc34 ubiquitin-conjugating enzyme plays a central role in progression of the cell cycle. Through analysis of the phenotype of a mutant missing a highly conserved sequence motif within the catalytic domain of Cdc34, we discovered previously unrecognized levels of regulation of the Ace2 transcription factor and the cyclin-dependent protein kinase inhibitor Sic1. In cells carrying the Cdc34^{tm} mutation, which alters the conserved sequence, the cyclin-dependent protein kinase inhibitor Sic1, an SCF^{Cdc4} substrate, has a shorter half-life, while the cyclin Cln1, an SCF^{Grr1} substrate, has a longer half-life than in wild-type cells. Expression of the *SIC1* gene cluster, which is regulated by Swi5 and Ace2 transcription factors, is induced in *CDC34^{tm}* cells. Levels of Swi5, Ace2, and the SCF^{Grr1} targets Cln1 and Cln2 are elevated in Cdc34^{tm} cells, and loss of Grr1 causes an increase in Ace2 levels. Sic1 levels are similar in *CDC34^{tm} ace2*Δ and wild-type cells, explaining a paradoxical increase in the steady-state level of Sic1 protein despite its reduced half-life. A screen for mutations that interact with *CDC34^{tm}* uncovered novel regulators of Sic1, including genes encoding the polyubiquitin chain receptors Rad23 and Rpn10.

UBIQUITIN is a small, 76-amino-acid residue protein. The covalent attachment of ubiquitin to another protein often serves as a signal for the selective degradation of that protein by the 26S proteasome (for review, see Glickman and Ciechanover 2002). Ubiquitylation is initiated by the ubiquitin-activating (or E1) enzyme, forming a high-energy thiolester intermediate with ubiquitin in an ATP-dependent reaction. The E1 transfers the ubiquitin molecule to a ubiquitin-conjugating (or E2) enzyme, which, like the E1, forms a thiolester with ubiquitin. The E2 then transfers ubiquitin onto a substrate. The transfer of ubiquitin to substrate typically requires the activity of a ubiquitin ligase (E3). The bond formed between ubiquitin and substrate is an isopeptide bond that links the COOH-terminal glycine residue of ubiquitin to the ε-amino group of a lysine residue of the substrate. Substrates may be monoubiquitylated or polyubiquitylated with the polyubiquitin chain being linked through one of the seven lysine residues within ubiquitin.

CDC34 was identified as a gene essential for cell viability and the initiation of DNA replication in the yeast *Saccharomyces cerevisiae* (Goebl *et al.* 1988). The Cdc34 protein is an E2 that ubiquitylates histones *in vitro* (Goebl *et al.* 1988) and itself *in vitro* (Banerjee *et al.* 1993) and *in vivo* (Goebl *et al.* 1994). Furthermore, Cdc34 genetically and physically interacts with the SCF family of ubiquitin ligases, and this interaction is a requirement for Cdc34 to carry out its ubiquitin-conjugating function *in vivo* (Mathias *et al.* 1998). Members of the SCF family of ubiquitin ligases are composed of at least four distinct proteins, including Skp1, Cdc53, Rbx1, and a member of a family of proteins known as F-box proteins. Rbx1 contains a ring-H2-finger domain and is essential for SCF-dependent attachment of ubiquitin to its substrates (Skowyra *et al.* 1999). Cdc53 is a cullin family member that binds both Skp1 and Cdc34. Skp1 tethers the F-box protein to the SCF complex. The F-box protein is the component of the SCF ligase that determines substrate specificity (for review, see Deshaies 1999). Cdc34 and SCF^{Cdc4} form an E2–E3 complex that mediates conjugation of lysine 48-linked polyubiquitin chains to the cyclin-dependent kinase inhibitor Sic1 as well the cell cycle regulator Far1 (Henchoz *et al.* 1997; Skowyra *et al.* 1997; Verma *et al.* 1997). On the other hand, SCF^{Grr1} is required for ubiquitylation of the cyclins Cln1 and Cln2 (Skowyra *et al.* 1997, 1999; Verma *et al.* 1997).

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.125302/DC1.

Microarray data from this article have been deposited with the NCBI GEO Data Libraries under accession nos. GSM210016–GSM210023.

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The Cdc34/Ubc7 family of ubiquitin-conjugating enzymes is defined by a conserved motif within the catalytic domain. This motif allows the Cdc34/Ubc7 family to catalyze both monoubiquitylation and ubiquitin chain extension (Petroski and Deshaies 2005). This motif is defined by two serines and a 12-amino-acid acidic loop, all of which lie in close physical proximity to the catalytic cysteine (Figure 1). In contrast, the majority of E2s, of which Rad6 is a classic example, have a lysine and aspartic acid residue in lieu of the serine residues and lack the acidic loop. Cdc34 mutants lacking the acidic loop monoubiquitylate Sic1 with the same kinetics as the wild-type enzyme but extend ubiquitin chains at a negligible rate *in vitro* (Petroski and Deshaies 2005). Cells expressing Cdc34 mutants that lack the acidic loop are inviable as are cells harboring S97D or S73K/S97 mutations (Liu *et al.* 1995). More recently, Li *et al.* (2007) found the acidic loop of the Cdc34-like ubiquitin-conjugating enzyme Ube2g1 to be essential for polyubiquitin chain preformation and conjugation to the known *in vivo* target HERPc, a short-lived, ER-associated protein. Interestingly, the polyubiquitin chain preformation and ubiquitin conjugation to HERPc *in vitro* require the presence of a RING-finger-containing protein, much like Cdc34 enzyme activity *in vivo*. During *in vitro* Cdc34 autoubiquitylation or histone ubiquitylation assays that do not require RING-finger-containing proteins, acidic loop deletion mutants function as well as, if not better than, Cdc34 (Pitluk *et al.* 1995; Varelas *et al.* 2003). Deletion of the acidic loop, residues 103–114, in combination with S73K and S97D mutations (hereafter referred to as the Cdc34 triple mutant, Cdc34^{tm}), restores full functionality to Cdc34 in all *in vitro* assays tested to date and *in vivo* as assessed by cell growth (Liu *et al.* 1995; Pitluk *et al.* 1995; Varelas *et al.* 2003). The goal of this study was to determine if processes and substrates controlled by Cdc34 are appropriately regulated in cells lacking this Cdc34 motif critical for polyubiquitin chain extension.

MATERIALS AND METHODS

Yeast strains: Standard methods were used for strain construction (Rose *et al.* 1990). Strains RC29, RRC73, RRC74, RRC76, and RRC78 containing the *CDC34^{tm}* allele flanked by the nourseothricin *N*-acetyltransferase gene were constructed as follows (Table 1). The plasmid pAG25 that contains the nourseothricin *N*-acetyltransferase gene (Goldstein and McCusker 1999) was amplified with adaptamer primers CDC34F2 (5′-ACTTTTTTCAAGGCTGAGAATCCATCGACA GATTGTAACGAAGCAGCTGAAGCTTCGTACGC-3′) and CDC34R2 (5′-TGCTCTGTATAGTTCAATAGAATCTTACAG TACATCACGCTGCAAGCATAGGCCACTAGTGGATCTG-3′) using the PCR cocktail and conditions described previously (Goldstein and McCusker 1999). The PCR product was transformed into the *CDC34^{tm}*-containing strain KS418 as previously described (Gietz and Woods 2002). Transformants were selected by plating cells on YPD plus 80 mg/liter of nourseothricin (Werner BioAgents, Jena, Germany). Insertion into the correct chromosomal locus was confirmed by PCR using the primers 34F2t (5′-CAAACTTGAGATGGAGTTGTTGA TG-3′) and pAG25Tr1 (5′-GTCAATCGTATGTGAATGCT-3′). This strain was named RC6. DNA containing the *CDC34^{tm}* allele and the *nat1* gene was amplified from RC6 genomic DNA with Phusion DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions using primers 34F2t and 34R3 (5′-ATGAGTAGTCGCAAAAG-CACCG-3′). For strains RRC73, RRC74, RRC76, and RRC78, the PCR product was then transformed into the appropriate BY4741 strain containing *CLN1-TAP*, *CLN2-TAP*, *SIC1-TAP*, or *FAR1-TAP* at the chromosomal locus as described (Ghaemmaghami *et al.* 2003). Transformation was carried out following the protocol of Johnston *et al.* (2002) for gene disruptions generated from a yeast genomic template. Transformants were selected by growth on YPD plus 80 mg/liter of nourseothricin.

RC29, the query strain for the synthetic gene array (SGA) screen, was made by a cross of RC6 and MT1901 to create the diploid RC21, which was sporulated and its tetrads dissected to isolate a haploid with the appropriate markers. RC94, the control strain for the secondary SGA screen, was constructed by insertion of the nourseothricin *N*-acetyltransferase gene into strain MT1901 at the exact chromosomal location as strain RC6. The PCR conditions and transformation were carried out as described for construction of RC6.

RC200 is a haploid made by crossing RC34 and BY4741 [SWI5-TAP(HIS3-MX6)]. Tetrads from the resulting diploid were dissected. RC34 was generated by dissection of the RC26 diploid, which resulted from a cross of strains RC6 and ResGen 11820 from the *Mat*α deletion library.

Media and growth conditions: Standard rich (YPD) and defined minimal (SD) media were prepared as described previously (Rose *et al.* 1990). For analysis of Far1 and Cln1 abundance and half-lives, cells were grown in YPD plus 30 mm succinic acid. Standard sporulation and dissection procedures were used as described previously (Rose *et al.* 1990).

For sulfite sensitivity assays, 2 mm of sodium sulfite and 75 mm of tartaric acid were added to YPD media as previously described (Avram and Bakalinsky 1996).

Western blotting: Cells were grown to a final density between 5×10^6 and 1.5×10^7 cells/ml. For the blots in Figure 3 and Figure 4A, cells were lysed by repeated cycles of vortex mixing with glass beads in breaking buffer containing 150 mm NaCl, 50 mm Tris–HCl, pH 7.5, 5 mm EDTA, 1% Triton X-100, 50 mm NaF, and one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) per 10 ml of breaking buffer. For Figure 4B, protein extract was made by the Horvath–Riezman method as previously described (Horvath and Riezman 1994). Briefly, 3.75 μl of extraction buffer [60 mm Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 5 mm EDTA, 50 mm NaF plus one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics) per 10 ml of extraction buffer] were added per milligram of wet cell pellet. Cells were resuspended in extraction buffer and boiled for 5 min. This mixture was then centrifuged, and the supernatant was saved as protein extract.

Lysates (~50 μg of protein) from either extraction method were loaded onto a 10% SDS-PAGE gel. Separated proteins were transferred to PVDF membrane. Western immunoblot analysis was performed by standard methods. Antibodies were used at the following dilutions: affinity-purified anti-Cdc34 (1:10,000), affinity-purified anti-Sic1 (1:1000), Ace2 antisera (1:5000) (Sbia *et al.* 2008), and anti-TAP (1:2000) (Open Biosystems). Primary antibody was detected with an HRP-conjugated goat anti-rabbit secondary antibody at a 1:10,000 dilution (Santa Cruz Biotechnology).

Half-life measurements: For measurement of Sic1 and Cln1 half-lives, strains Sic1-Tap, Cln1-Tap, RRC73, and RRC74 were

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
2690	*MAT***a** *sic1::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
4080	*MAT***a** *swi5::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
4088	*MAT***a** *ace2::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
7137	*MAT***a** *haa1::Kan^R his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
BY4741-(Cln1Tap)	*MAT***a** *CLN1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ghaemmaghami *et al.* (2003)
BY4741-(Cln2Tap)	*MAT***a** *CLN2-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ghaemmaghami *et al.* (2003)
BY4741-(Far1Tap)	*MAT***a** *FAR1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ghaemmaghami *et al.* (2003)
BY4741-(Sic1Tap)	*MAT***a** *SIC1-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ghaemmaghami *et al.* (2003)
BY4741-(Swi5Tap)	*MAT***a** *SWI5-TAP(HIS3-MX6) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Ghaemmaghami *et al.* (2003)
DBY2059	*MATα leu2-3,112*	K. Hennessey
KS415	*MAT***a** *grr1::URA3 ura3-52 leu2Δ-1 his3Δ-200*	Schweitzer *et al.* (2005)
KS418	*MAT***a**, *CDC34^{tm} ura3 leu2 trp1 lys2 ade2 ade3*	This study
KS422	*MAT***a** *ura3 leu2 trp1 lys2 ade2 ade3*	Schweitzer *et al.* (2005)
MT1901	*MATα mfa1Δ::pMFA1-HIS3 can1Δ ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	M. Tyers
RC21	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ura3/ura3 leu2/leu2 trp1/TRP1 lys2/LYS2 ade2/ADE2 ade3/ADE3 his3/HIS3 MFA1/mfa1Δ::pMFA1-HIS3 can1/CAN1*	This study
RC29	*MATα CDC34^{tm}(NAT1) mfa1Δ::pMFA1-HIS3 his3Δ ura3Δ leu2Δ can1Δ*	This study
RC94	*MATα CDC34(NAT1) mfa1Δ::pMFA1-HIS3 his3Δ1 leu2Δ0 ura3Δ0 can1Δ*	This study
RC96	*MAT***a** *CDC34^{tm}(NAT1) ace2Δ::Kan^R pMFA1-HIS3 ura3Δ0 leu2Δ0 can1*	This study
RC100	*MAT***a** *CDC34^{tm}(NAT1) swi5Δ::Kan^R pMFA1-HIS3 ura3Δ0 leu2Δ0 can1 met15Δ0*	This study
RC106	*MAT***a**/α *CDC34(NAT1)/CDC34 rad23::Kan^R/RAD23 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC113	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 rad23::Kan^R/RAD23 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC131	*MAT***a**/α *CDC34(NAT1)/CDC34 ctk2::Kan^R/CTK2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC141	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ctk2::Kan^R/CTK2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC166	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 rad23::Kan^R/RAD23 sic1::URA3/SIC1 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC167	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ctk2::Kan^R/CTK2 sic1::URA3/SIC1 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC168	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ctk2::Kan^R/CTK2 ace2::URA3/ACE2 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC169	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ctk2::Kan^R/CTK2 swi5::URA3/URA3 mfa1Δ::pMFA1-HIS3/MFA1 his3/his3 ura3/ura3 leu2/leu2 can1/CAN1 met15/MET15*	This study
RC171	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 ubp14::Kan^R/UBP14 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC172	*MAT***a**/α *CDC34(NAT1)/CDC34 ubp14::Kan^R/UBP14 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC173	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 cka2::Kan^R/CKA2 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study

(*continued*)

TABLE 1
(Continued)

Strain	Genotype	Reference
RC174	*MAT***a**/α *CDC34(NAT1)/CDC34 cka2::Kan^R/CKA2 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC175	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 rps7b::Kan^R/RPS7B mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RC176	*MAT***a**/α *CDC34(NAT1)/CDC34 rps7b::Kan^R/RPS7B mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RRC43	*MAT***a**/α *CDC34^{tm}(NAT1)/CDC34 rim13::Kan^R/RIM13 mfa1Δ::pMFA1-HIS3/MFA1 his3Δ/his3Δ ura3/ura3Δ0 leu2/leu2Δ0 can1/CAN1 met15Δ0/MET15*	This study
RRC73	*MAT***a** *CLN1-TAP(HIS3-MX6) CDC34^{tm}(NAT1) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC74	*MAT***a** *SIC1-TAP(HIS3-MX6) CDC34^{tm}(NAT1) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC76	*MAT***a** *FAR1-TAP(HIS3-MX6) CDC34^{tm}(NAT1) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RRC78	*MAT***a** *CLN2-TAP(HIS3-MX6) CDC34^{tm}(NAT1) his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
RC200	*MAT*α *SWI5-TAP(HIS3-MX6) CDC34^{tm}(NAT1) his3Δ1 leu2Δ0 ura3Δ0*	This study
RC34	*MAT*α *cdc34tm(NatR) vhs3ΔKanR ura3 leu2 lys2 his3*	This study
RRC85	*MAT*α *CDC34^{tm}(NAT1)*	This study
YL10-1	*MAT***a** *cdc34-2 leu2Δ1 ura3-52 trp1Δ63 his3Δ* Gal+	Liu *et al.* (1995)

inoculated into 150 ml of YPD + 30 mM succinic acid (pH 4.5) grown at 30° until mid-log phase at which time cycloheximide (0.42 mg/ml final concentration) was added. Thirty-milliliter samples were collected at the indicated time points and immediately frozen in liquid nitrogen. Protein extraction was accomplished as described in *Western blotting*. Immunoreactivity quantification was accomplished using Quantity One (BioRad) software, where the ratio of the adjusted volume of the Sic1 or Cln1 band was normalized to the adjusted volume of the Cdc34 band.

Microarray analysis: A *CDC34^{tm}* strain (RRC85) and an isogenic wild-type strain (DBY2059) were inoculated from stationary-phase cultures into a synthetic defined medium containing 2% dextrose, 0.17% yeast nitrogen base minus amino acids and ammonium sulfate, 0.25% L-glutamine, 0.025% magnesium sulfate, and 25.2 mg/liter L-leucine. Four separate cultures of each strain were grown at 30°, allowing between three and four doublings, and cells were collected at ~8×10^6 cells/ml. Cells were centrifuged and immediately frozen in liquid nitrogen. Total RNA was extracted using a hot acid phenol-chloroform protocol as previously described (Schmitt *et al.* 1990). RNA quality was verified with OD260/280 readings and agarose gel electrophoresis (1.5%). An Affymetrix Genechip Scanner 3000 and Affymetrix Yeast 2.0 arrays were used for the microarray. A Student's *t*-test of the log base 2-transformed data was used to establish *P*-values for Table 2 and Table 3.

Synthetic gene array: Duplicate SGA screens of the *CDC34^{tm} (nat1)* query strain, RC29, were performed against the ~4700 single, nonessential gene deletion strains as previously described (Tong *et al.* 2001). Computer-based image analysis of colony size allowed genetic interactions to be scored on a scale of 1–4 with 1 = lethal, 2 = severely sick, 3 = sick, and 4 = mildly sick. Candidate synthetic genetic interactions were validated by a second SGA screen, which was done manually. Strains RC29 and RC94 were separately crossed to strains with the candidate gene deletion. RC94 is isogenic to RC29 except the *nat1* gene marks wild-type *CDC34*. Diploids were selected by replica-plating onto YPD + 100 mg/liter nourseothricin + 200 mg/liter G418 and then isolated by streaking for single-cell colonies on the same media. The isolated diploids were sporulated on sporulation media (1% potassium acetate, 2% agar, 0.1% yeast extract, 0.05% glucose supplemented with 1 mM uracil, L-histidine, and L-leucine). Diploids were sporulated at room temperature for 7 days and then struck onto haploid, double-mutant selection media (2% glucose, 0.17% yeast nitrogen base lacking ammonium sulfate and amino acids, 0.1% monosodium L-glutamic acid, 0.2% amino acid mix lacking L-histidine and L-arginine, 50 mg/liter canavanine, 100 mg/liter nourseothricin, 200 mg/liter G418, and 2% agar). Each *CDC34^{tm}*(*nat1*) *xxxΔ::KanR* genetic interaction was scored visually after 3 days growth at 30° by comparing its growth to that of the *CDC34*(*nat1*) *xxxΔ::KanR* haploid.

To determine if deletion of *SIC1*, *ACE2*, or *SWI5* was capable of suppressing the lethality of certain double mutants, we replaced each gene with the *Kluyveromyces lactis URA3* gene in a diploid strain that was heterozygous for the gene deletion of interest (for example, *RAD23*) and heterozygous for the *CDC34^{tm}* allele. The primers used were as follows: *SIC1* (CCAC CAAGGTCCAGAGGGACTAGGTACCTTACTAGTGGATCTG ATATCAC and GCTCTTGATCCCTAGATTGAAACAATGCCT CGATTTAGGTGACACTAT), *ACE2* (TGGATAACGTTGTAGA TCCGTGGTATATAAACTAGTGGATCTGATATCAC and TCA GAGAGCATCAGTTTCGTTTGAAAGGGTGCGATTTAGGTG ACACTAT), and *SWI5* (GATGGATACATCAAACTCTTGGTTT GATGCACTAGTGGATCTGATATCAC and CTTTGATTAGTT TTCATTGGCGAAACCATAC-GATTTAGGTGACACTAT). The plasmid pUG72 (Gueldener *et al.* 2002), which contains the

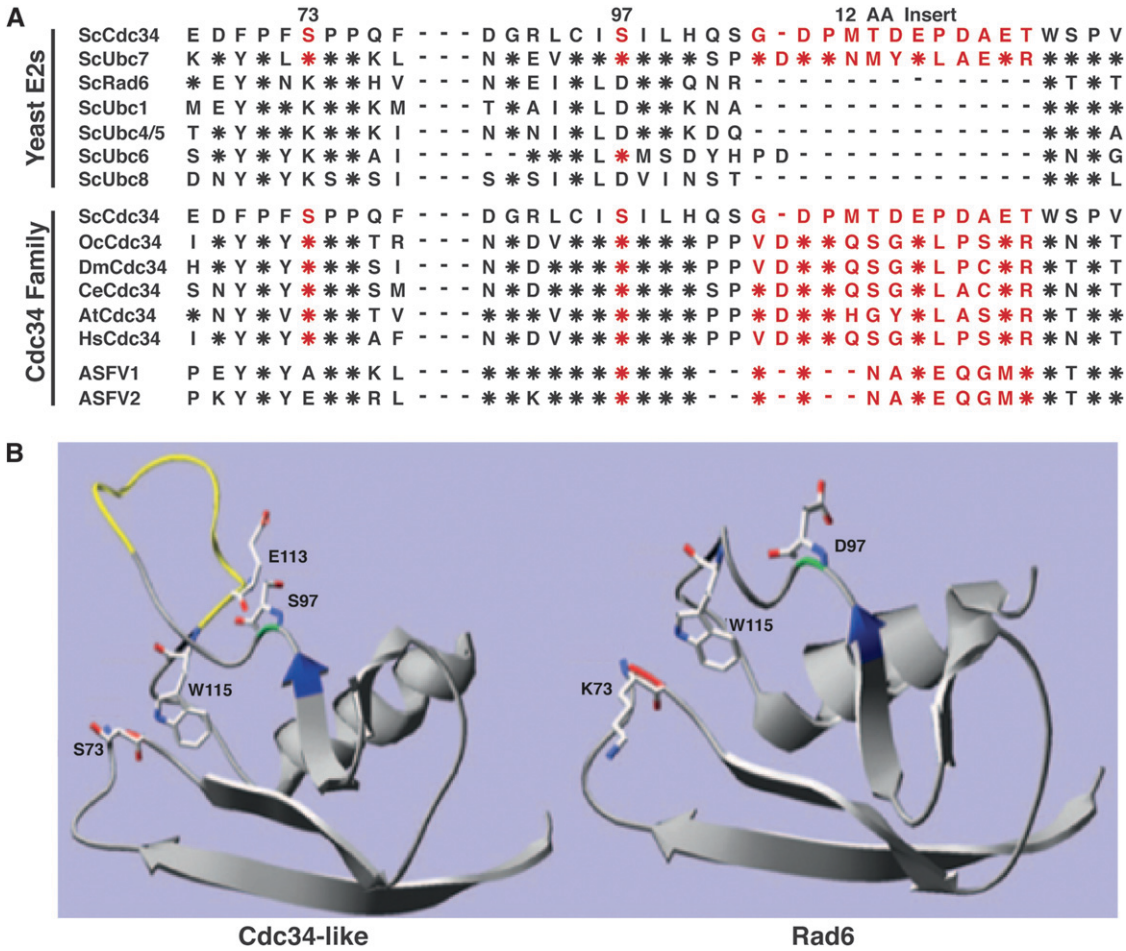

FIGURE 1.—Structure and alignment of E2s: The serine/serine/loop motif is conserved in all Cdc34 family members. (A) Partial alignment of yeast E2s and Cdc34/Ubc7 family members. Red indicates amino acid residues unique to the Cdc34 family of E2s (the regulatory triad). Asterisks represent identities unique to Cdc34. Dashes represent gaps. Sc, *S. cerevisiae*; Oc, *Oryctolagus cuniculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; ASFV1, African swine fever virus (GI:9628248); ASFV2, African swine fever virus (GI:450743). (B) (Left) Structure of Ubc7 (COOK *et al.* 1997; Protein Data Bank identifier "2UCZ") depicting amino acids Asp51-Ser128. The blue arrow represents the catalytic cysteine. The serines corresponding to Cdc34 S73 and Cdc34 S97 of the catalytic triad are labeled, and amino acid residues Gly103-Arg114, which constitute the insert, are yellow. The invariant residue Trp110 and the highly conserved residue Asp108 of the loop are depicted in atomic detail to demonstrate proximity to each serine. (Right) A stereo view of Rad6 (WORTHYLAKE *et al.* 1998; Protein Data Bank identifier "1AYZ") depicting amino acids Asp50-Asn114. The catalytic cysteine is blue and amino acid residues equivalent to Cdc34 Ser73 and Cdc34 Ser97 are labeled and shown in atomic detail.

K. lactis URA3 gene, was amplified with adaptamers for *SIC1*, *ACE2*, or *SWI5* listed above using the PCR cocktail and conditions described previously (GOLDSTEIN and MCCUSKER 1999). The PCR product was transformed into the desired diploids as previously described (GIETZ and WOODS 2002). Transformants were isolated and patched onto YPD and grown for 24 hr after which cells were replica-plated onto the sporulation media described above. Diploids were sporulated at room temperature for 7 days and then struck onto the haploid, double-mutant selection media described above. Plates were imaged after 72 hr of incubation at 30°.

RESULTS

A partial sequence alignment of the yeast ubiquitin-conjugating enzymes and Cdc34 orthologs is shown in Figure 1. It is apparent that there is a relationship among S73, S97, and a 12-residue loop region that surrounds the catalytic cysteine. Ubc7, like Cdc34, contains serine residues at the positions equivalent to yeast Cdc34 S73 and S97. In contrast, the majority of E2s, of which Rad6 is a typical example, have a lysine and an aspartic acid residue, respectively, at these positions. Cdc34/Ubc7 family members also contain a loop near S97 (residues 103–114 of Cdc34) that other E2s lack. One of these two alternative motifs of serine/serine/loop or lysine/aspartic acid/loopless is present in most eukaryotic E2s (LIU *et al.* 1995). The amino acid residues that constitute the motif are in close physical proximity. The crystal structure for Ubc7 shows serine residue S91 (Cdc34 S97 equivalent) to lie within 3 Å of the well-

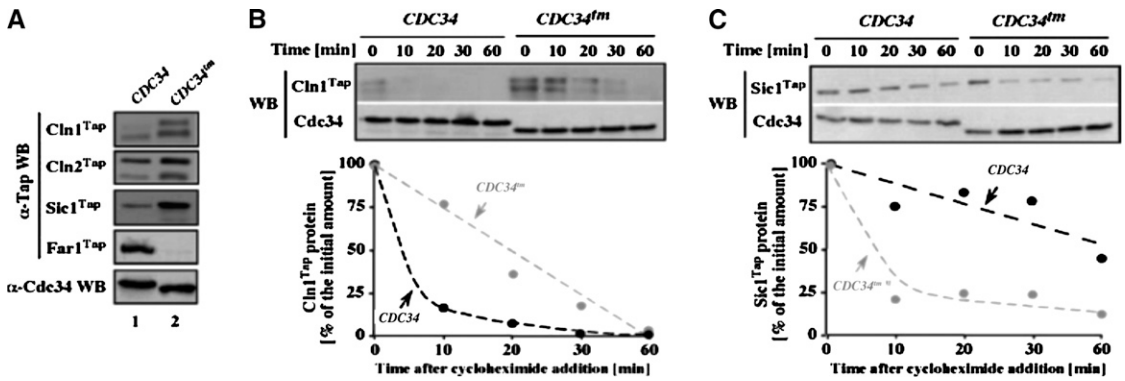

FIGURE 2.—Replacement of *CDC34* with the *CDC34*^{tm} allele leads to unexpected changes in steady-state levels and half-lives of substrates. (A) Protein levels were analyzed by α-Tap Western blot of extracts made from haploid cells containing a genomic copy of either *CDC34* (GHAEMMAGHAMI *et al.* 2003) or *CDC34*^{tm} (strains RRC74, RRC76, RRC73, RRC78). At least in the case of the Sic1^{Tap} construct, its cell cycle fluctuations were confirmed to be identical to untagged Sic1 (GHAEMMAGHAMI *et al.* 2003). As in the case of Cln1 and Cln2 (TYERS *et al.* 1993), two bands are visible in the α-Tap Cln^{Tap} Western blots, representing different electrophoretic mobilities of the phosphorylated and unphosphorylated forms. Comparable levels of Cdc34 and Cdc34^{tm} proteins were detected by α-Cdc34 Western blot in each type of an extract, but only the analysis of the Far1^{Tap} extract is shown. (B and C) Protein levels were analyzed as in A, except that extracts were prepared from mid-log-phase yeast cultures harvested at various times after cycloheximide treatment as indicated. Graphs show quantitation of the Western blot results, as indicated.

conserved aspartic acid residue (D113) of the 12-amino acid loop while serine residue S67 (Cdc34 S73 equivalent) is within 4 Å of an invariant tryptophan residue (W115) (COOK *et al.* 1997). Even with differences at these amino acid residues, the tertiary fold of the Cdc34/Ubc7 family resembles that of the Rad6 family (Figure 1B).

The Cdc34-loopless protein has been evaluated in SCF-dependent *in vitro* reactions (PETROSKI and DESHAIES 2005). In the absence of the loop region, the monoubiquitylation of Sic1 occurs much as with wild-type Cdc34; however, both chain formation and the specificity of the lysine-48 linkage are compromised. Cdc34^{tm} has yet to be investigated in an SCF-dependent *in vitro* reaction. There are important distinctions that can be made between *CDC34*$^{\Delta 103\text{-}114}$ and *CDC34*^{tm}. We have shown that the *CDC34*$^{\Delta 103\text{-}114}$ mutation supports growth much more poorly than either *CDC34* or *CDC34*^{tm} and not at all when expressed from a weak promoter (LIU *et al.* 1995). However, *CDC34*^{tm} does not notably compromise growth, viability, or cell cycle distribution, even as a precise ORF replacement into the *CDC34* chromosomal locus (LIU *et al.* 1995; data not shown).

***CDC34*^{tm} differentially affects the abundance and half-life of SCF substrates:** The disparity between the less efficient, Cdc34$^{\Delta 103\text{-}114}$-catalyzed Sic1 polyubiquitylation and the lack of a notable change in growth and viability in *CDC34*^{tm} strains led us to test whether cells dependent upon Cdc34^{tm} had different steady-state levels of known Cdc34 substrates *in vivo*. Strikingly, the levels of Cln1, Cln2, Sic1, and Far1 are altered in a *CDC34*^{tm} strain (Figure 2A). The steady-state abundances of Sic1, Cln1, and Cln2 are increased while the Far1 steady-state level is reduced in the *CDC34*^{tm}-expressing strain. Consistent with previous studies, Cln1 protein is visualized as two bands with the slower-migrating species known to represent a modified species (TYERS *et al.* 1993). A similar pattern of migration is seen when yeast extracts are probed for Cln2 protein and the slower-migrating species are attributable, at least in part, to phosphorylation (WITTENBERG *et al.* 1990).

We measured the half-lives of Sic1 and Cln1 to determine whether the increased abundance of these proteins in the *CDC34*^{tm} strain reflected an increase in protein stability. Half-life measurements reveal Sic1 to be much less stable while Cln1 is more stable in *CDC34*^{tm} cells (Figure 2, B and C), a difference that is not attributable to an alteration in cell cycle distribution as assessed by flow cytometric analysis of DNA content and budding index (data not shown). In wild-type cells, the Sic1 half-life is ~50 min, and this measurement agrees with previous measurements of Sic1 half-life (BAILLY and REED 1999). Strikingly, the Sic1 half-life in *CDC34*^{tm}-expressing cells is reduced to <10 min. While the half-life of Cln1 is <10 min in wild-type cells, the Cln1 half-life in the *CDC34*^{tm} strain is nearly 20 min. This is double the half-life in wild-type cells but not as long as the Cln1 half-life (~60 min) in *cdc34* temperature-sensitive cells (BLONDEL and MANN 1996). Thus the SCF^{Grr1} substrates Cln1 and Cln2 are stabilized in *CDC34*^{tm} cells while the SCF^{Cdc4} substrate Sic1 is destabilized. We cannot address the stability of the SCF^{Cdc4} substrate Far1 as it becomes undetectable in *CDC34*^{tm} cells. However, a likely explanation for this Far1 effect is a decrease in its stability.

An increase in the mRNA level of *SIC1* and a coregulated gene cluster in *CDC34*^{tm} cells can be attributed to the transcription factor Ace2: To investigate the basis of the increase in Sic1 steady-state

TABLE 2

SIC1* cluster of cell cycle genes is upregulated by *CDC34^{tm}

ORF	Alias	Fold change	*P*-value	Transcription factor
YDR055W	*PST1*	−2.17	7.9*e*-05	Slight Ace2/Swi5
YKL163W	*PIR3*	−1.35	0.058111	Slight Ace2/Swi5
YJL159W	*HSP150*	−1.1	0.001441	Slight Ace2/Swi5
YNR067C	*DSE4*	−1.06	0.635923	Not detected
YBR083W	*TEC1*	−1.04	0.897662	Not affected
YKL116C	*PRR1*	1.14	0.04453	Not detected
YNL192W	*CHS1*	1.16	0.063773	Not affected
YGR086C	*PIL1*	1.18	0.023168	Not affected
YDL117W	*CYK3*	1.21	0.052732	Not detected
YJL078C	*PRY3*	1.25	0.217825	Not detected
YNL327W	*EGT2*	1.34	0.003588	Ace2/Swi5
YBR158W	*AMN1*	1.34	0.000267	Ace2/Swi5
YIL009W	*FAA3*	1.38	0.001426	Not affected
YKL185W	*ASH1*	1.38	0.000885	Ace2/Swi5
YKL164C	*PIR1*	1.41	7.2*e*-05	Swi5
YJL194W[a]	*CDC6*	1.41	0.04	Swi5
YDL127W[a]	*PCL2*	1.47	0.03	Swi5
YNL078W	*NIS1*	1.51	0.005773	Swi5
YDL179W	*PCL9*	1.52	0.000748	Swi5
YGR044C	*RME1*	1.54	0.001677	Ace2/Swi5
YOR264W	*DSE3*	1.6	0.000353	Ace2/Swi5
YLR079W	*SIC1*	1.63	0.000288	Ace2/Swi5
YLR286C	*CTS1*	1.8	2.3*e*-05	Ace2
YPL158C	—	1.87	6.1*e*-05	Swi5
YHR143W	*DSE2*	1.89	5.8*e*-05	Ace2
YNL046W[a]	—	2.13	5.00*e*-06	Swi5
YDL227C[a]	*HO*	2.2	9.20*e*-05	Swi5
YGL028C	*SCW11*	2.58	1.0*e*-06	Ace2
YER124C	*DSE1*	3.61	5.0*e*-06	Ace2

The genes that are coregulated through the cell cycle with *SIC1* as defined by Spellman *et al.* (1998) are shown with their relative expression levels and *P*-values in a *CDC34^{tm}* strain relative to an isogenic wild type as determined by microarray analysis. The column "Transcription factor" is derived from a separate study that determined the influence of the two main transcription factors of this gene cluster, Ace2 and Swi5 (Doolin *et al.* 2001). The genes are arranged in order of their relative expression.

[a] Not originally identified to be coregulated with Sic1 (Spellman *et al.* 1998) but defined as being Swi5 or Ace2 dependent (Doolin *et al.* 2001).

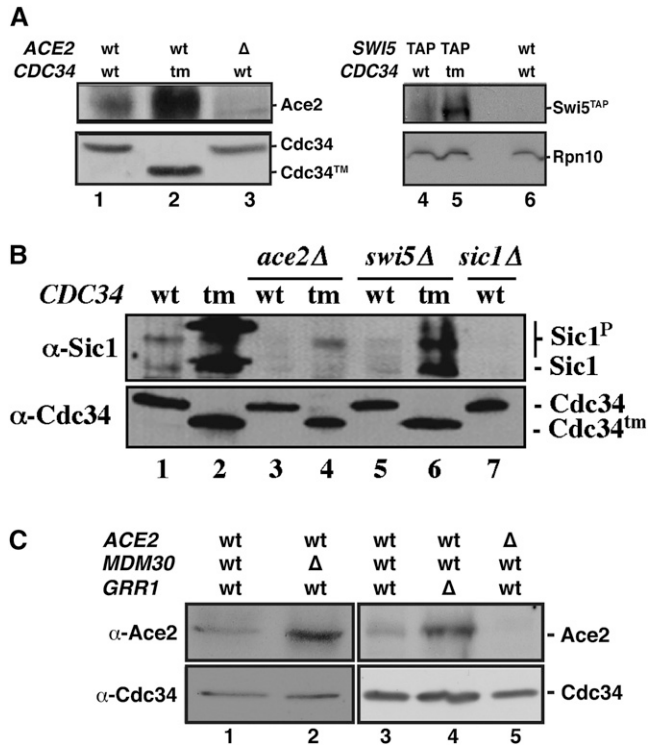

Figure 3.—Ace2 is responsible for the increase in Sic1 steady-state abundance in *CDC34^{tm}* cells and is a potential substrate of both SCF^{Grr1} and SCF^{Mdm30}. (A and C) Protein extracts were prepared by the Horvath–Riezman method from yeast of the indicated genotype and analyzed with affinity-purified α-Ace2, α-TAP, α-Rpn10, or α-Cdc34 antibodies, as indicated. (B and C) Protein extracts were prepared from yeast of the indicated genotype and analyzed with α-Sic1, α-Ace2, or α-Cdc34 antibodies, as indicated.

abundance in *CDC34^{tm}* cells and to better understand the functional significance of the Cdc34/Ubc7-specific motif (serine73/serine97/loop), microarray analysis was performed. Four biological replicates were sampled for the *CDC34^{tm}* strain and an isogenic wild-type strain. *FAR1* and *CLN2* mRNA levels are not significantly different in *CDC34^{tm}* cells. However, *CLN1* and *SIC1* mRNA levels are significantly increased (*P*-value < 0.01) in the *CDC34^{tm}* strain. Examination of the cluster of genes coregulated throughout the cell cycle with *SIC1*, as defined by Spellman *et al.* (1998), revealed that the expression of >60% of the genes in this cluster is significantly increased (*P*-value < 0.05) (Table 2). Again, this effect cannot be attributed to an altered cell cycle distribution of the *CDC34^{tm}* strain as asynchronous *CDC34^{tm}* cultures have a cell cycle distribution approximating that of wild-type cultures as assessed by DNA content (data not shown). The transcriptional flux of most genes in this cluster is attributed to the transcriptional activities of Ace2 and Swi5. Ace2 and Swi5 share highly similar DNA-binding domains and regulate many of the same genes (Butler and Thiele 1991; Dohrmann *et al.* 1996). However, certain genes, such as *HO* and *CTS1*, depend solely on either Swi5 or Ace2 for their expression (Dohrmann *et al.* 1992; McBride *et al.* 1999). Swi5 and Ace2 have both overlapping and independent roles in the regulation of the *SIC1* coregulated gene cluster (Doolin *et al.* 2001). As shown in Table 2, Ace2-dependent genes are the most highly upregulated in *CDC34^{tm}* cells. Furthermore, *CLN3* transcription is repressed by Ace2 in daughter cells, and this is part of the mechanism that allows daughter cells to grow to a larger size prior to START, the cell cycle commitment point (Laabs *et al.* 2003; Di Talia *et al.* 2009). *CLN3* mRNA levels are decreased 1.41-fold (*P*-value = 0.006) in *CDC34^{tm}* cells.

TABLE 3

Genes induced in response to acetaldehyde, including targets of the transcription factor Haa1, are repressed in *CDC34*^{tm} cells

ORF	Alias	Cdc34tm fold change	Cdc34tm *P*-value	Transcription factor	Acetaldehyde fold change
YPR157W	—	−8.78	2.60*e*-05	Haa1	101.01
YER037W	*PHM8*	−8.68	0.005485	Haa1	8.54
YLR297W	—	−3.38	0.000164	Haa1	7.86
YIR035C[a]	—	−2.74	4.00E-06	Haa1	Not Detected
YPR156C[a]	*TPO3*	−2.73	0.002466	Haa1	Not Detected
YBR054W	*YRO2*	−17.55	0.00065	Haa1/Hsf1	14.23
YER150W	*SPI1*	−3.67	0.051281	Haa1/Hsf1	78.26
YNL160W	*YGP1*	−3.39	0.004359	Haa1/Hsf1	5.51
YGR138C	*TPO2*	−3.84	0.000148	Haa1/Sok2	111.65
YER130C	—	−3.05	2.20*E*-05	Haa1/Sok2	6.05
YCR021C	*HSP30*	−34.85	0.000473	Hsf1	284.03
YDR171W	*HSP42*	−20.84	0.005181	Hsf1	20.58
YJL144W	—	−15.75	0.005232	Hsf1	48
YGR249W	*MGA1*	−8.04	0.005973	Hsf1	9.01
YGR142W	*BTN2*	−7.81	0.055734	Hsf1	35.6
YGR248W	*SOL4*	−7.33	0.022398	Hsf1	14.6
YDR258C	*HSP78*	−6.27	0.042359	Hsf1	6.4
YOR134W	*BAG7*	−6.24	0.00011	Hsf1	4.31
YFL053W	*DAK2*	−5.17	0.013542	Hsf1	7.83
YPR158W	—	−4.33	0.006109	Hsf1	4.48
YPR015C	—	−3.78	0.000101	Hsf1	4.87
YGL037C	*PNC1*	−3.72	0.006157	Hsf1	3.46
YLL026W	*HSP104*	−3.53	0.055282	Hsf1	12.71
YNL077W	*APJ1*	−3.46	0.008297	Hsf1	5.7
YJL082W	*IML2*	−3.45	2.10*E*-05	Hsf1	3.69
YBR214W	*SDS24*	−3.41	0.007507	Hsf1	5.48
YPL247C	—	−2.66	0.007523	Hsf1	3.2
YNL007C	*SIS1*	−2.55	0.004259	Hsf1	3.77
YOR267C	*HRK1*	−2.38	0.000483	Hsf1	4.96
YFL040W	—	−2.32	0.000625	Hsf1	4.33
YOL032W	*OPI10*	−2.25	0.025852	Hsf1	5.19
YBR101C	*FES1*	−1.99	0.005175	Hsf1	6.11
YER035W	*EDC2*	−2.55	0.002572	Hsf1/Sok2	4.76
YHL021C	—	−2.37	0.015077	Hsf1/Sok2	7.59
YGR088W	*CTT1*	−1.94	0.005252	Hsf1/Sok2	10.08
YER028C	*MIG3*	−4.48	0.000248	Sok2	7.18
YNR014W	—	−4.31	0.000868	Sok2	3.26
YMR316W	*DIA1*	3.31	0.001082	Sok2	10.62
YER053C	*PIC2*	−3.21	0.046544	Sok2	12.13
YOL016C	*CMK2*	−2.39	0.035474	Sok2	28.58
YGL179C	*TOS3*	−2.14	0.00326	Sok2	3.4
YLR121C	*YPS3*	−2	0.009871	Sok2	3.02
YOR298C-A	*MBF1*	−2	0.010964	Sok2	3.09
YDL038C	—	−5.51	1.00*E*-05	Sok2/Mga1	4.68
YDL048C	*STP4*	−4.91	1.00*E*-05	Sok2/Mga1	6.18
YKL043W	*PHD1*	−2.16	0.008645	Sok2/Mga1	11.51
YDR259C	*YAP6*	−2.1	0.000163	Sok2/Mga1	4.76
YOR273C	*TPO4*	−5.23	0.002478	Mga1	6.5
YBR183W	*YPC1*	−3.08	0.001528	Mga1	3.6
YMR181C	—	−2.64	1.20*E*-05	???	4.15
YOL014W	—	−2.39	0.006914	???	13.86
YFR022W	*ROG3*	−2.33	1.00*E*-06	???	10.53
YLR343W	*GAS2*	−2.27	0.000181	???	8.48
YPL165C	*SET6*	−2.25	0.000919	???	4.41
YGR008C	*STF2*	−2	0.112446	???	4.32

The genes that are both repressed in *CDC34*^{tm} cells (this work) and induced in *CDC34* cells in response to exogenous acetaldehyde (Aranda and Del Olmo 2004) are shown with their relative expression level (fold change) and *P*-value. Rows are ordered according to the respective transcription factor(s), which was determined by querying the YEASTRACT database (http://www.yeastract.com). ???, transcription factor(s) unknown.

[a] Targets of the transcription factor Haa1 that were not detected in response to acetaldehyde.

These microarray results suggested that in *CDC34*^{tm} cells either Ace2 or Swi5 activity is increased. Since known Cdc34 targets include transcription factors, we hypothesized that an increase in either Ace2 or Swi5 abundance is responsible for the increase in *SIC1* mRNA levels. To address this hypothesis, Western blot analysis was performed to examine Ace2 and Swi5 levels in wild-type and *CDC34*^{tm} cells. As seen in Figure 3A, both Ace2 and Swi5 protein levels are elevated in *CDC34*^{tm} cells as compared to wild-type cells. The increase in steady-state protein level arises post-transcriptionally since the microarray analysis reveals no significant difference in the mRNA levels of *ACE2* or *SWI5* between wild-type and *CDC34*^{tm} cells. We next examined the level of Sic1 protein in *CDC34*^{tm} cells in the absence of *ACE2* and *SWI5*. Disruption of *ACE2* in the context of *CDC34*^{tm} reduces Sic1 protein levels to below that detected in the wild-type strain; on the other hand, deletion of *SWI5* in *CDC34*^{tm} cells has little effect on the steady-state abundance of Sic1 (Figure 3B). In these experiments (Figure 3B), a rapid protein extraction method using SDS and heat was utilized for the analysis of Sic1 protein (compare with Figure 2). This method allows for the detection of protein modifications such as phosphorylation and ubiquitylation. Under these extraction conditions, Sic1 is visualized as two differently migrating species with the lower band migrating at the size observed when other extraction conditions are employed (see Figure 2). The slower-migrating species is likely Sic1 containing phosphorylation or ubiquitylation modification(s) and, importantly, is not visualized in *sic1*Δ protein extracts. Thus, the increase in the steady-state abundance of Sic1 in *CDC34*^{tm} cells is due to increased Ace2-dependent transcription of *SIC1*, which most likely results from an increase in Ace2 protein abundance.

As the increased steady-state abundance of Ace2 in *CDC34*^{tm} cells is due to a post-transcriptional mechanism, we postulated that Ace2 is targeted for degradation by an SCF/Cdc34 complex. Therefore, the steady-state abundance of Ace2 was assessed in strains lacking each of the genes encoding F-box proteins (namely *SAF1*, *DIA2*, *MDM30*, *GRR1*, *HRT3*, *UFO1*, *RCY1*, *DAS1*, *MFB1*, *ELA1*, *YMR258c*, *YLR352w*, *YDR306c*, *YNL311c*, *YDR131c*, and *YLR224w*) except the essential genes *CDC4* and *MET30*. Ace2 levels remain unchanged in all strains tested except *grr1*Δ and *mdm30*Δ cells, which both have increased levels of the Ace2 protein (Figure 3C, lanes 2 and 4; supporting information, Figure S1). This is consistent with the finding that both Cln1 and Cln2, SCF^{Grr1} substrates, accumulate in *CDC34*^{tm} cells and with our conclusion that the *CDC34*^{tm} strain is partially defective in degradation of SCF^{Grr1} substrates.

***CDC34*^{tm} is not a hypomorphic allele—targets of the transcription factor Haa1 are downregulated in *CDC34*^{tm} cells:** A previous microarray comparing *cdc34* and *cdc53* temperature-sensitive strains found an increase in the activity of the Met4, Gcn4, and Tec1 transcription factors (Varelas *et al.* 2006). Met4, Gcn4, and Tec1 are SCF substrates, and their stabilization in the *cdc34* and *cdc53* temperature-sensitive strains leads to increased activation of their respective target genes. The analysis of *CDC34*^{tm} cells did not show a comparable change in Met4-, Gcn4-, or Tec1-dependent transcripts (Table S1). However, 57 gene transcripts were significantly (P-value < 0.05) upregulated at least twofold, and 140 were significantly downregulated by at least twofold in *CDC34*^{tm} cells (Table S1). To determine the transcription factor(s) whose activity is altered in *CDC34*^{tm} cells, we analyzed our data using the YEASTRACT (http://www.yeastract.com) database, which relates *S. cerevisiae* transcriptional regulators and their target genes (Teixeira *et al.* 2006). Ninety percent of documented Haa1 target genes are downregulated more than twofold in *CDC34*^{tm} cells (Table 3). Haa1 is critical for the transcriptional response to exogenous acetaldehyde and weak organic acids. Although insensitive to exogenous acetaldehyde, *haa1*Δ strains are sensitive to acetic and sorbic acid (Aranda and del Olmo 2004; Fernandes *et al.* 2005).

Elevated levels of acetaldehyde can inhibit the growth of *S. cerevisiae* cells (Stanley *et al.* 1993). Excess acetaldehyde can be detoxified by allowing it to react with sulfite to form acetaldehyde hydroxysulfonate (Casalone *et al.* 1992). Acetaldehyde hydroxysulfonate production can also mitigate the growth inhibitory effects of excess sulfite, which can be present exogenously or produced during the intracellular reduction of sulfate to cysteine (Casalone *et al.* 1992). Microarray studies demonstrate that part of the response to acetaldehyde exposure is an increase of intracellular sulfite levels via upregulation of the homocysteine production pathway and reduction of the sulfite exporter Ssu1 (Aranda and del Olmo 2004). Strains lacking transcriptional regulators of the methionine biosynthetic pathway (*MET4*, *CBF1*, and *MET28*) are sensitive to 1 g/liter levels of acetaldehyde.

We compared the *CDC34*^{tm} transcriptional profile to an acetaldehyde response (Aranda and del Olmo 2004) and discovered a strong inverse correlation. Many of the transcripts most highly induced by acetaldehyde are significantly downregulated in a *CDC34*^{tm} strain (Table 3). Conversely, *SSU1*, which encodes the sulfite extrusion pump, is downregulated 3.3-fold in cells exposed to acetaldehyde but is upregulated 3-fold in *CDC34*^{tm} cells. Genes encoding the branch points of glucose fermentation that would shunt glucose flux away from acetaldehyde production, namely the NAD-dependent glycerol-3-phosphate dehydrogenases *GPD1* and *GPD2*, are both downregulated ~3-fold while genes encoding the first and second steps of the pentose phosphate pathways, namely *ZWF1* (−2.2) and *SOL4* (−7.3), are also both decreased in *CDC34*^{tm} cells (Figure 4A).

The inverse correlation between cells exposed to acetaldehyde and *CDC34*^{tm} cells led us to consider whether *CDC34*^{tm} cells would be sensitive to sulfite because of an

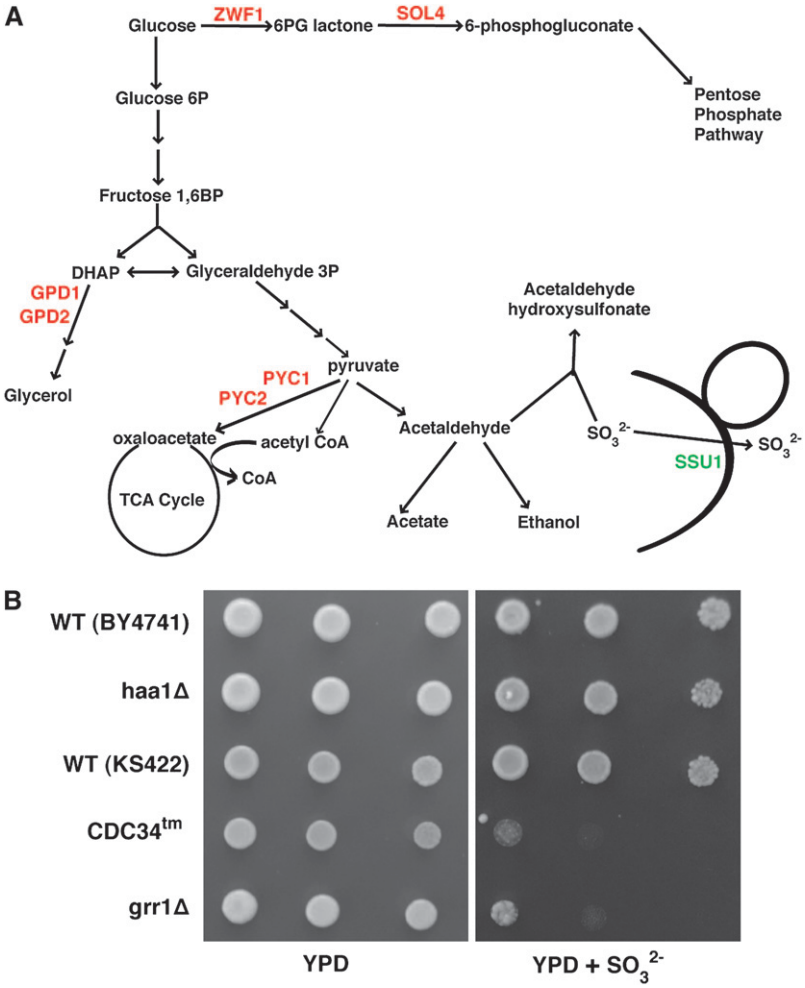

FIGURE 4.—mRNAs encoding enzymes that reduce the metabolic flux through glycolysis are reduced in *CDC34^{tm}* cells and correlate with *CDC34^{tm}* and *grr1*Δ strain sulfite sensitivity. (A) Metabolic pathways of glycolysis and its shunts. The mRNAs that are significantly reduced (*P*-value < 0.01) in *CDC34^{tm}* cells are in red. mRNAs that do not significantly differ between wild-type and *CDC34^{tm}* cells are not shown. The mRNA encoding the sulfite extrusion pump, *SSU1*, is significantly upregulated (*P*-value < 0.01) in *CDC34^{tm}* cells and is shown because it directly affects acetaldehyde levels by limiting the formation of acetaldehyde hydroxysulfonate. (B) Strains BY4741 (wild type), 7137 (*haa1*Δ), KS422 (wild type), KS418 (*CDC34^{tm}*), and KS415 (*grr1*Δ) were grown overnight, adjusted to equal densities, serially diluted 10-fold, spotted onto YPD plates (pH 3.5) either with or without 2 mM sulfite, and incubated at 37° for 2 days.

inability to detoxify it. Indeed, *CDC34^{tm}* cells are much more sensitive than their isogenic wild-type counterparts (Figure 4B). It has previously been shown that *grr1*Δ cells are sensitive to sulfite (AVRAM and BAKALINSKY 1996), which again supports the conclusion that Cdc34^{tm} compromises SCF^{Grr1} activity. Thus the reduction in Haa1 targets in *CDC34^{tm}* cells likely reflects reduced intracellular acetaldehyde.

***CDC34^{tm}* cell viability requires polyubiquitin receptors of the 26S proteasome and the RNA Pol II kinase CTDK-I to regulate Ace2 and Sic1:** As an alternative approach to identifying the functional significance of the Cdc34/Ubc7-specific motif, an SGA screen using the *CDC34^{tm}* allele as the query gene was performed. This was accomplished by tightly linking the *CDC34^{tm}* allele to the nourseothricin *N*-acetyltransferase gene (*nat1*) from *Streptomyces noursei*, which confers nourseothricin (*nat1*) resistance (GOLDSTEIN and MCCUSKER 1999). The *nat1* start codon was placed ~1700 nucleotide bases 5′ of the *CDC34tm* start codon. As expected, the *nat1* gene faithfully segregated with the *CDC34^{tm}* allele in >20 individual segregants derived from a *CDC34^{tm}::nat1/CDC34* diploid (data not shown). The SGA screen was carried out in duplicate, and genetic interactions were scored by computer-based image analysis of colony size (Figure 5A). To our knowledge, this is the first synthetic lethal screen accomplished using a neomorphic mutant rather than a loss-of-function gene deletion. Notably, 14 genes proximal to the *CDC34* chromosomal locus scored as synthetically lethal in the primary screen. Reduced recombination between neighboring loci prevents facile generation of double-mutant haploids. Genes proximal to *CAN1* and the *MFA1::pMFA1-HIS3* loci also appeared in the primary screen. These interactions result from reduced recombination frequency rather than from true synthetic lethality and were not included in the secondary screen.

Candidate genetic interactions from the primary SGA screen were confirmed by crossing both a *CDC34^{tm}::nat1* strain and a *CDC34::nat1* strain to strains carrying deletions of genes that scored as synthetically lethal in the primary screen (Figure 5A). Diploids were struck onto media that selected for haploids with both *nat1* (marking either *CDC34* or *CDC34^{tm}*) and *KanR* (marking interacting gene deletion). To our knowledge, this is the first time that the control strain, which determines the gene deletions that are lethal when crossed to a wild-

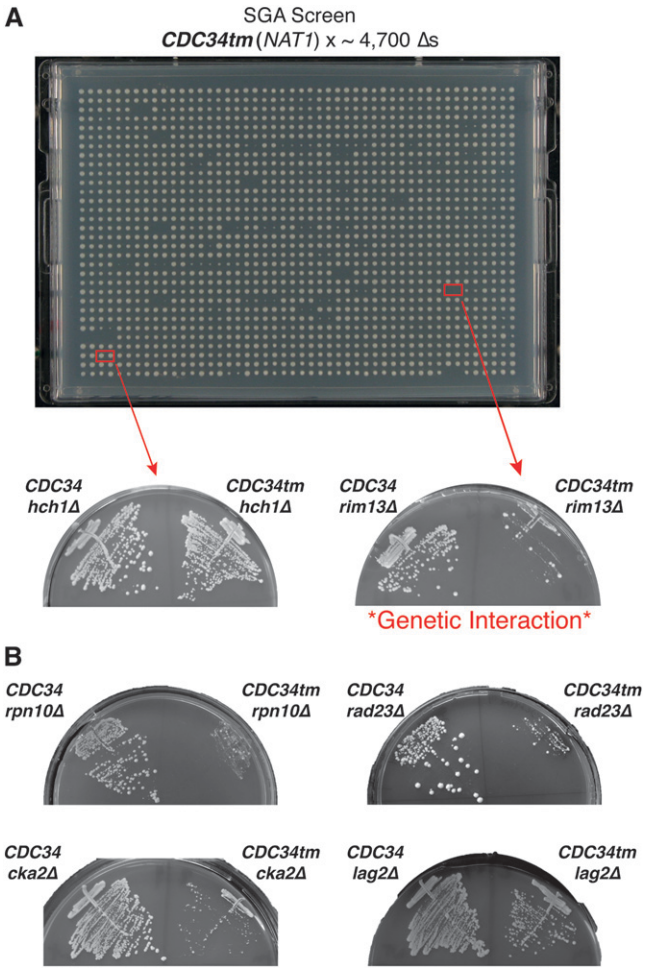

FIGURE 5.—Synthetic lethality analysis identifies several groups of genes that are critical for growth of *CDC34*^{tm} but not of *CDC34* yeast. (A) General schematic of our SGA approach. An example of results of the primary (top) and secondary (bottom) screen using the *CDC34*^{tm} query allele or the control *CDC34* allele, as indicated, each marked with *nat1* in the same location (for details, see text and MATERIALS AND METHODS). (B) Haploids of the indicated genotype were selected by streaking sporulated, heterozygous diploids on haploid selection media plus G418 and nourseothricin.

type strain, was noted to harbor the resistance gene in exactly the same genomic location as the query strain. In a typical synthetic lethal screen, *nat1* marks the gene deletion rather than being present at the same position as in the control strain.

In all, 88 genes were confirmed to be either synthetically lethal or sick with *CDC34*^{tm} (Table 4). Rpn10 is a non-ATPase subunit of the 19S regulatory particle of the proteasome that binds medium-to-long K48-linked polyubiquitin chains. Deletion of *RPN10* stabilizes Sic1 (VERMA *et al.* 2004). The human Rpn10 ortholog, S5a, was the first 26S proteasome subunit implicated in the binding of polyubiquitylated substrates (DEVERAUX *et al.* 1994). Deletion of *RAD23* is also synthetically lethal with *CDC34*^{tm} (Figure 5B). Rad23 has a ubiquitin-like domain (UBL) near its N terminus and a ubiquitin associated (UBA) domain that binds K48-linked ubiquitin chains *in vitro* (BERTOLAET *et al.* 2001). In yeast, Rad23 can bind substrates with shorter polyubiquitin chains (four to six ubiquitin molecules) through its UBA domain and recruit them to the proteasome through an interaction between its UBL domain and Rpn1 (ELSASSER *et al.* 2002). Yeast strains lacking *RAD23* are not impaired in their ability to degrade Sic1 *in vivo*, but 26S proteasomes isolated from *rad23*Δ strains are defective in Sic1 deubiquitylation and degradation *in vitro* (VERMA *et al.* 2004). The synthetic lethality between *CDC34*^{tm} and deletion of either *RPN10* and *RAD23* is particularly intriguing because utilization of polyubiquitin receptors in different contexts might explain the ability of *CDC34*^{tm} cells to survive while the polyubiquitin-conjugating activity of the SCF complex is compromised as suggested by *in vitro* reconstitution of SCF ubiquitylation using the Cdc34$^{\Delta 103\text{-}114}$ mutant (PETROSKI and DESHAIES 2005). Dsk2 and Ddi1 are also short-chain polyubiquitin receptors of the 26S proteasome and, like Rad23, have both UBL and UBA domains. Neither Dsk2 nor Ddi1 appeared in the initial *CDC34*^{tm} synthetic lethal screen, and we do not detect a fitness defect in *CDC34*^{tm} *ddi1*Δ or *CDC34*^{tm} *dsk2*Δ haploids (data not shown).

Two known regulators and an interacting partner of the SCF complexes were identified as synthetically lethal with *CDC34*^{tm}. *CKA2*, one of two catalytic subunits of Casein Kinase II, phosphorylates any of six serines within the acidic C terminus of Cdc34, and it is synthetically lethal with *CDC34*^{tm} (Figure 5B). Phosphorylation of Cdc34 by Casein Kinase 2 increases its activity against Sic1 (SADOWSKI *et al.* 2007; COCCETTI *et al.* 2008). Furthermore, Casein Kinase 2 also phosphorylates Ser201 of Sic1, which promotes Sic1 degradation (COCCETTI *et al.* 2006; TRIPODI *et al.* 2007). Another regulator of the SCF complex, *LAG2*, shares a genetic interaction with *CDC34*^{tm} (Figure 5B). Lag2 negatively regulates the SCF by binding to and preventing neddylation of Cdc53 (LIU *et al.* 2009; SIERGIEJUK *et al.* 2009). It is interesting that *CDC34*^{tm} has negative genetic interactions with both positive and negative regulators of Cdc34, further supporting the hypothesis that *CDC34*^{tm} is a neomorphic mutant. *CDC34*^{tm} is also synthetically lethal with *PRB1*, which encodes the vacuolar proteinase B. Multiple studies have revealed a physical interaction between Prb1 and components of the SCF, including Cdc53, Rbx1, and Skp1 (Ho *et al.* 2002; KATO *et al.* 2010). Although it is not clear at this time how Prb1 interacts with SCF/Cdc34, the abundance of physical interactions with the SCF complex and its genetic interaction with *CDC34*^{tm} suggest a physiologically relevant relationship. The discovery of known Cdc34 regulators/interactors raises the intriguing possibility that some of the other genes sharing negative genetic interactions with *CDC34*^{tm} play a role in Cdc34/SCF regulation.

TABLE 4

***CDC34^{tm}* genetic interactions with nonessential genes**

Gene product function	Alias
Cell growth regulation	*FPR1*,[a] *RAS2*,[a] *RIM13*,[b] *RIM9*,[b] *RIM101*,[b] *RIM20*,[b] *RIM8*,[b] *SOK2*,[b] *TIP41*[b]
Cell stress	*HAL1*,[b] *NRG2*,[a] *RIM13*,[b] *RIM9*,[b] *RIM101*,[b] *RIM20*,[b] *RIM8*,[b] *SOY1*,[a] *TIR1*[b]
Chromatin	*AHC1*,[a] *CTK2*,[b] *CTK1*,[b] *CTK3*,[b] *YAF9*,[b] *IES2*,[b] *IES5*,[b] *HHT1*[a]
Cytoskeleton	*BNI4*,[a] *LSB3*,[a] *SPH1*[a]
DNA damage	*MRE11*,[b] *RAD16*,[b] *RAD30*,[b] *RAD51*[b]
Metabolism	*ABZ1*,[b] *ADE12*,[b] *COQ6*,[b] *COX17*,[a] *OPI3*,[a] *PGM3*,[a] *SIP1*[a]
Metal homeostasis	*FSF1*,[b] *PPZ2*[a]
Microtubule function	*CIK1*,[b] *DYN1*,[a] *DYN2*,[b] *GIM5*,[b] *KAR3*[a]
Protein trafficking	*SEC22*,[a] *VAC8*,[b] *VPS21*,[b] *VPS45*,[a] *SWA2*,[a] *GVP36*,[a] *NIR1*,[a] *GET2*,[b] *CHS5*,[a] *MNN11*,[a] *YUR1*,[a] *PIB2*,[a] *YIP2*[a]
Signaling-phosphorylation	*BNI4*,[a] *CKA2*,[b] *CTK1*,[b] *CTK2*,[b] *CTK3*,[b] *FPR1*,[a] *PPZ2*,[a] *DBF2*,[a] *SIP1*,[a] *TIP41*,[b] *TPK2*,[a] *TPK3*[a]
Signaling-Phosphatidyl inositol	*PDR16*,[a] *PDR17*,[a] *PIB2*[a]
Transcription	*CTK1*,[b] *CTK2*,[b] *CTK3*,[b] *NRG2*,[a] *RIM13*,[b] *RIM9*,[b] *RIM101*,[b] *RIM20*,[b] *RIM8*,[b] *RTR2*,[a] *SOK2*,[b] *STB1*[a]
Translation	*DOM34*,[a] *GCN1*,[b] *NCS2*,[b] *RPL16B*,[a] *RPL34A*,[a] *RPL34B*,[a] *RPL29*,[b] *TIF4631*[a]
Ubiquitin pathway	*DFM1*,[b] *LAG2*,[a] *RAD23*,[b] *RPN10*,[b] *UBP14*[b]
Miscellaneous	*FCY21*,[a] *NUP100*,[a] *PEX13*,[b] *PRB1*[b]
Unknown	*YCL049c*,[a] *YEL043w*,[b] *YHL042w*,[b] *YHR151c*,[a] *YMR102c*,[b] *YNL034w*,[b] *YNR070w*[a]

These genes were confirmed to share a negative genetic interaction with *CDC34^{tm}*.
[a] Limited growth.
[b] No growth.

The three genes encoding proteins of the CTDK-I complex (Ctk1, Ctk2, and Ctk3) are synthetically lethal with *CDC34^{tm}* (Figure 6, A and B; data not shown). The CTDK-1 complex coordinates transcriptional elongation, pre-mRNA 3′-end processing, and translational fidelity (reviewed in Hampsey and Kinzy 2007). The role of CTDK-1 in transcriptional elongation is well characterized, and its ability to phosphorylate the second serine of the repetitive C-terminal domain of the largest subunit of Pol II (Rpo21) increases the efficiency of transcriptional elongation (Lee and Greenleaf 1997; Patturajan *et al.* 1999). *CDC34^{tm} ctk2*Δ haploids die as multiple, attached cells indicative of a cell separation defect (Figure 6C). As Ace2 is required for the expression of *CTS1* and cell separation, we hypothesized that misregulation of Ace2 and some of its transcriptional targets was the cause of the *CDC34^{tm} ctk2*Δ lethality. Interestingly, removal of *ACE2*, *SIC1*, or *SWI5* is able to suppress the lethality of a *CDC34^{tm} ctk2*Δ mutant. These results suggest that the lethal defect of the *CDC34^{tm} ctk2*Δ strain is increased production of Sic1 that is alleviated by loss of the *SIC1* activators Ace2 and Swi5 or by loss of *SIC1* itself (Figure 6D).

DISCUSSION

This work originated with the discovery that mutations modifying Cdc34 serine residue 97 or deleting amino acid residues 103–114 in the N-terminal catalytic domain make a nonfunctional enzyme, but a combination of mutations at these positions (and S73) yields a functional enzyme, as assessed by *in vivo* complementation of a *cdc34* null strain. However, recent work has shown that the stretch of acidic residues in Cdc34 is necessary for timely and appropriate polyubiquitin chain extension of Sic1 *in vitro* (Petroski and Deshaies 2005). Therefore, we reasoned that since a strain bearing the Cdc34$^{\Delta 103\text{-}114}$ mutant does not support growth while a Cdc34^{tm} strain is viable and has a growth rate comparable to wild type, there must be *in vivo* mechanisms that compensate for the defect in polyubiquitin chain extension. In many cases, multiple ubiquitin-conjugating enzymes must function together to polyubiquitylate a single substrate, with one E2 serving to monoubiquitylate and the other extending the ubiquitin chain (Rodrigo-Brenni and Morgan 2007). However, no genetic interaction between *CDC34^{tm}* and any of the other ubiquitin-conjugating enzymes was detected in the primary SGA screens, suggesting that other E2s are not compensating for the defective polyubiquitylation activity of the Cdc34^{tm}. In contrast, *CDC34^{tm}* exhibits strong genetic interactions with the polyubiquitin receptor genes *RAD23* and *RPN10*. Rad23 and Rpn10 have been shown to be required for Sic1 degradation; however, neither is an essential gene. A *rad23*Δ *rpn10*Δ strain degrades Sic1 more slowly than strains bearing either single mutation, but is viable. This argues for an additional mechanism of recruiting Sic1 to the proteasome for degradation (Lambertson *et al.* 1999; Verma *et al.* 2004). Both *CDC34^{tm} rad23*Δ and *CDC34^{tm} rpn10*Δ cells have multiple, elongated buds (data not shown) reminiscent of *cdc34^{ts}* cells at 37° and indicative of defective Sic1 degradation. *CDC34^{tm} rpn10*Δ and *CDC34^{tm} rad23*Δ mutants are not rescued by simultaneous deletion of *SIC1* (data not shown), but this is not altogether surprising since *cdc34^{ts} sic1*Δ cells progress through START but arrest in mitosis as large-budded cells.

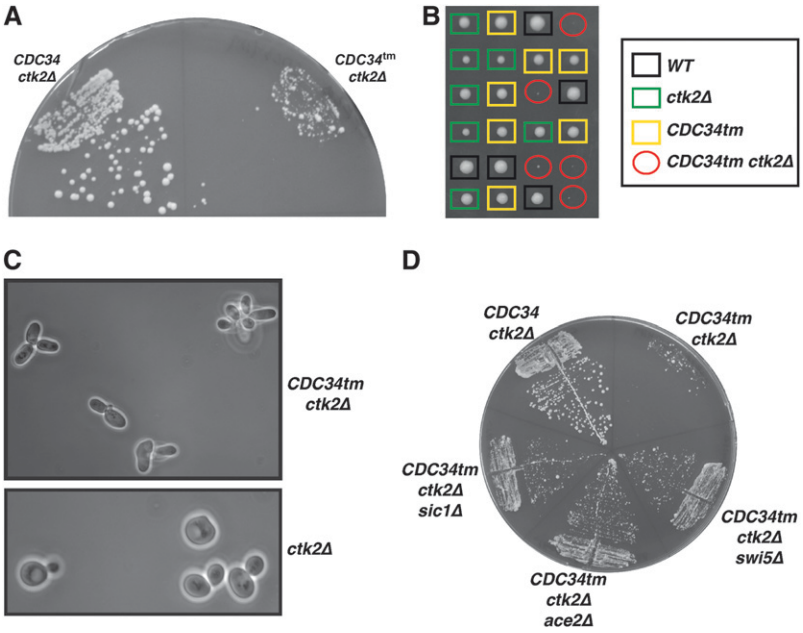

FIGURE 6.—*CTK2* is synthetically lethal with *CDC34*^{tm}. (A) Haploids with the indicated genotypes were selected by streaking heterozygous diploids on haploid selection media with G418 and nourseothricin. Plates were incubated at 30° for 3 days. (B) A *CDC34*^{tm}/*CDC34 ctk2*Δ/*CTK2* diploid, RC141, was sporulated and dissected on YPD. (C) Haploids of the indicated genotype were isolated by tetrad dissection and imaged microscopically. (D) Diploid strains with the indicated genotypes (Table 1) were sporulated and then struck on a haploid double-mutant selection plate that allows for growth of only the indicated haploid. Plate was incubated at 30° for 3 days.

The SCF^{Cdc4} substrate Sic1 has a shorter half-life in *CDC34*^{tm} as compared to wild-type cells. We were unable to determine the half-life of Far1 in *CDC34*^{tm} cells because it is undetectable. On the other hand, the SCF^{Grr1} substrate Cln1 is more stable in *CDC34*^{tm} cells compared to wild-type cells. If steady-state abundance of the Cln proteins is indicative of activity, Cln/CDK activity should be increased in *CDC34*^{tm} cells. The Cln/Cdc28 complexes phosphorylate Sic1 and Far1 and, on the basis of the number of CDK sites, likely phosphorylate Cln1 as well (MENDENHALL *et al.* 1987; PETER *et al.* 1993; HENCHOZ *et al.* 1997). We postulate that increased Cln/Cdk phosphorylation of the substrates Sic1, Far1, Cln1, and Cln2 enables substrate recruitment to the individual SCF complexes. Upon encountering a Cdc34^{tm}-bound SCF complex, the substrate is ubiquitylated, but with a polyubiquitin chain that may be different from that formed by wild-type Cdc34. This could mean that in Cdc34^{tm} mutants the time between SCF association and dissociation may be shorter for each substrate and thereby available for proteasomal degradation earlier than for substrates that encounter a wild-type Cdc34-containing SCF complex. Alternatively, the polyubiquitin receptors necessary for substrate proteasomal recognition may be different for each of these substrates when ubiquitylated by Cdc34^{tm}. Our data do not reveal the contribution of each step (phosphorylation, ubiquitylation, proteasome binding) to the overall differences in substrate steady-state abundance and half-lives in *CDC34*^{tm} cells. However, our data suggest that Cdc34^{tm} be considered a tool for both *in vivo* and *in vitro* experiments as we continue to dissect the contribution of each step to the rate of the entire process.

An interesting paradox is seen in the simultaneous reduction in Sic1 protein half-life and increase in the steady-state abundance of the Sic1 protein in a *CDC34*^{tm} strain due to increased Ace2 activity. It is tempting to consider the possibility that Ace2 is an SCF^{Grr1} and SCF^{Mdm30} substrate. The SCF ubiquitin ligase complexes are responsible for the ubiquitylation of numerous transcription factors. Recent evidence demonstrates that ubiquitylation of these substrates can have both a positive and a negative role in transcriptional regulation but does not always result in proteasome-mediated degradation (LIPFORD *et al.* 2005; CHANDRASEKARAN and SKOWYRA 2008). Gal4, a transcription factor required for *GAL* gene induction, is ubiquitylated by SCF^{Grr1} and SCF^{Mdm30} (MURATANI *et al.* 2005). SCF^{Grr1} targets an inactive pool of Gal4 for proteasome-mediated degradation under non-inducing conditions. SCF^{Mdm30} targets the transcriptionally active pool of Gal4 for proteolysis. Mdm30-dependent degradation of transcriptionally active Gal4 stimulates cotranscriptional mRNA processing of the *GAL* gene mRNAs (MURATANI *et al.* 2005). In addition, Gal4 transcriptional activity is enhanced by Gal4 monoubiquitylation. The 19S regulatory particle of the 26S proteasome binds to promoter-bound Gal4 and dissociates the Gal4–DNA complex (FERDOUS *et al.* 2007). When Gal4 is monoubiquitylated, it is resistant to the dissociating activity of the 19S regulatory particle.

We measured the half-life of Ace2 in wild-type and *CDC34*^{tm} cells but found no detectable difference (data not shown). However, the transcription of *ACE2* and localization of Ace2 are regulated in a cell cycle-dependent manner. *ACE2* mRNA is upregulated during the G2/M transition, and Ace2 enters both the mother cell nucleus and the daughter cell nucleus late in mitosis (DOHRMANN *et al.* 1992). Ace2 is preferentially retained in the daughter nucleus when phosphorylated by the daughter-specific Mob2/Cbk1 kinase complex (COLMAN-LERNER *et al.* 2001; WEISS *et al.* 2002; MAZANKA *et al.* 2008). We suspect that separately measuring the

half-life of Ace2 within the daughter nucleus and the mother and daughter cytoplasm will be more informative regarding the mechanism by which Ace2 is upregulated in *CDC34^{tm}*, *grr1*Δ, and *mdm30*Δ cells.

The synthetic lethality of the *ctk2*Δ *CDC34^{tm}* strain can be suppressed by deletion of *ACE2*, *SIC1*, or *SWI5*. This finding suggests that the CTDK-1 complex negatively regulates Sic1 activity by affecting Swi5 and Ace2 activity toward *SIC1*. Swi5 is known to be targeted for proteasome-mediated degradation by SCF^{Cdc4}. Phosphorylation of at least one of the eight consensus CDK sites targets Swi5 to SCF^{Cdc4}. The Srb10 cyclin-dependent kinase is partially responsible for phosphorylation of Swi5, but it is not the sole kinase involved. Swi5 is stabilized in a *srb10*Δ strain but not nearly to the same degree as in a strain in which all eight CDK consensus sites of Swi5 have been removed by serine-to-alanine mutations (Kishi *et al.* 2008). We propose that Ctk1 is also involved in targeting Swi5 to SCF^{Cdc4}. Both Srb10 and Ctk1 phosphorylate the CTD of RNA Pol II. Therefore, these kinases are often associated with the transcriptional machinery, and thus both Swi5 and Ace2 may at times be in close physical proximity to these kinases. The fact that deletion of *SIC1*, *SWI5*, or *ACE2* suppresses the lethality of *CDC34^{tm} ctk2*Δ cells suggests that the CTDK-I complex is involved in the regulation of both Swi5 and Ace2.

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New Insight Into the Role of the Cdc34 Ubiquitin-Conjugating Enzyme in Cell Cycle Regulation via Ace2 and Sic1

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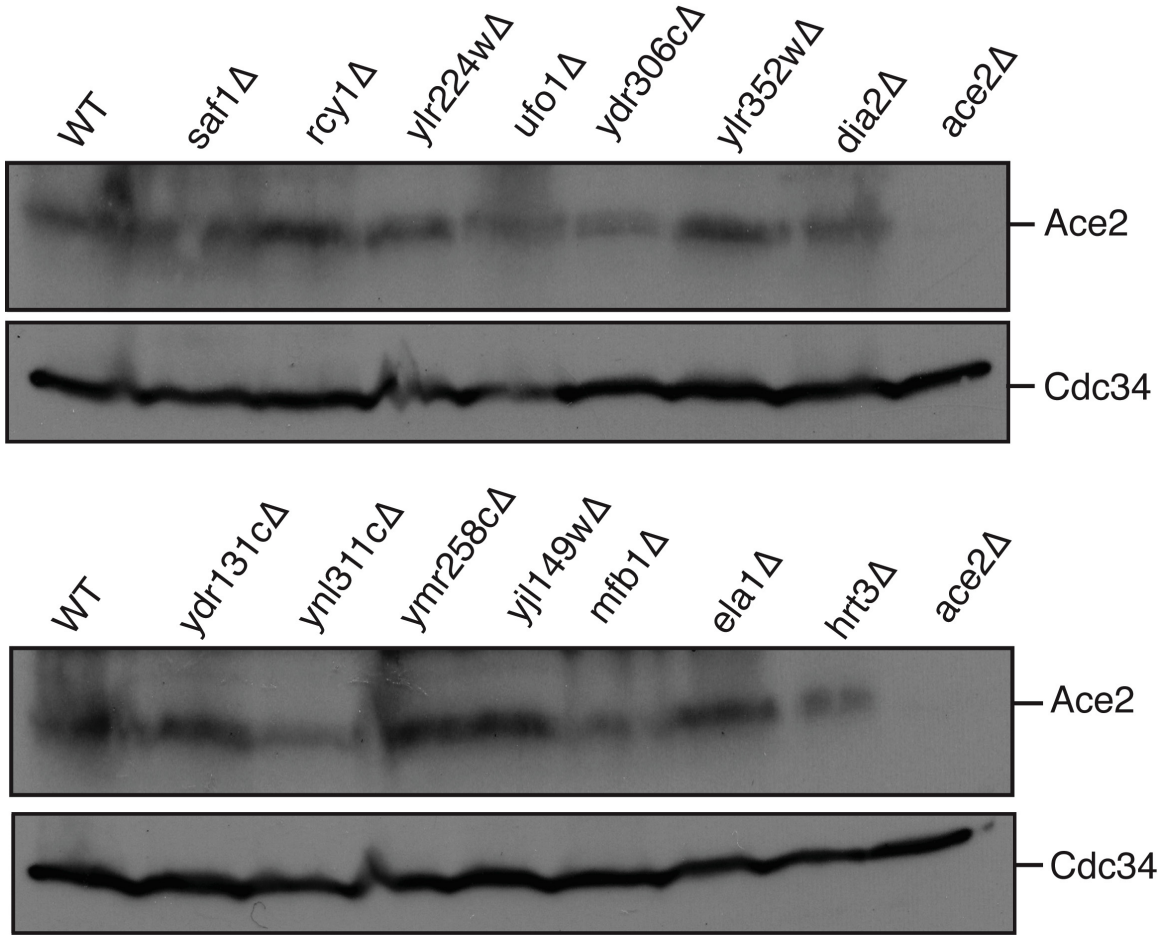

FIGURE S1.—Ace2 steady state protein levels are unaffected by deletion of most genes encoding F-box proteins. Protein extracts were prepared from yeast of the indicated genotype and analyzed with α-Ace2 or affinity purified α-Cdc34 antibodies, as indicated.

TABLE S1

Comparative microarray analysis of CDC34tm and wild type strains

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.125302/DC1.