The Role of Cdh1p in Maintaining Genomic Stability in Budding Yeast

Karen E. Ross and Orna Cohen-Fix[1]

The Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Manuscript received December 13, 2002
Accepted for publication June 11, 2003

ABSTRACT

Cdh1p, a substrate specificity factor for the cell cycle-regulated ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C), promotes exit from mitosis by directing the degradation of a number of proteins, including the mitotic cyclins. Here we present evidence that Cdh1p activity at the M/G_1 transition is important not only for mitotic exit but also for high-fidelity chromosome segregation in the subsequent cell cycle. *CDH1* showed genetic interactions with *MAD2* and *PDS1*, genes encoding components of the mitotic spindle assembly checkpoint that acts at metaphase to prevent premature chromosome segregation. Unlike *cdh1*Δ and *mad2*Δ single mutants, the *mad2*Δ *cdh1*Δ double mutant grew slowly and exhibited high rates of chromosome and plasmid loss. Simultaneous deletion of *PDS1* and *CDH1* caused extensive chromosome missegregation and cell death. Our data suggest that at least part of the chromosome loss can be attributed to kinetochore/spindle problems. Our data further suggest that Cdh1p and Sic1p, a Cdc28p/Clb inhibitor, have overlapping as well as nonoverlapping roles in ensuring proper chromosome segregation. The severe growth defects of both *mad2*Δ *cdh1*Δ and *pds1*Δ *cdh1*Δ strains were rescued by overexpressing Swe1p, a G_2/M inhibitor of the cyclin-dependent kinase, Cdc28p/Clb. We propose that the failure to degrade cyclins at the end of mitosis leaves *cdh1*Δ mutant strains with abnormal Cdc28p/Clb activity that interferes with proper chromosome segregation.

CELL cycle progression must be carefully regulated to preserve genome integrity. In addition to the many proteins that carry out the structural and mechanical aspects of duplicating and segregating chromosomes, an extensive network of regulatory proteins oversees these events. The fidelity of chromosome segregation is ensured, in part, by the spindle assembly checkpoint that regulates the metaphase-to-anaphase transition (reviewed in Gardner and Burke 2000). Kinetochores that lack bipolar attachments to the mitotic spindle send out a signal that is recognized and transmitted by the checkpoint proteins Mad1p, Mad2p, Mad3p, Bub1p, Bub3p, and Mps1p. The signal itself is not well understood, but it probably stems from a kinetochore not occupied by microtubules and/or absence of tension on an unattached or mono-attached kinetochore (Zhou *et al.* 2002). In budding yeast the end result of the checkpoint signaling pathway is stabilization of the anaphase inhibitor, Pds1p. This is achieved by inhibiting a ubiquitin ligase, the anaphase-promoting complex/cyclosome (APC/C), which in conjunction with Cdc20p is required to promote Pds1p's ubiquitination and subsequent degradation (Visintin *et al.* 1997). Pds1p blocks anaphase initiation by binding to and inhibiting the separase Esp1p, so named because it promotes separation of sister chromatids through cleavage of a cohesin subunit, Scc1p (Ciosk *et al.* 1998; Uhlmann *et al.* 1999). Once all chromosomes have established bipolar attachments to the spindle, the checkpoint signal ceases and Pds1p is ubiquitinated and degraded, releasing Esp1p to promote anaphase initiation. Under normal conditions, neither the checkpoint pathway nor Pds1p is essential, but if spindle assembly or kinetochore function is compromised both the checkpoint and Pds1p become essential (Hoyt *et al.* 1991; Li and Murray 1991; Yamamoto *et al.* 1996a). Pds1p plays a similar role in response to DNA damage, where the activation of the DNA damage checkpoint pathway results in Pds1p's stabilization (Yamamoto *et al.* 1996b; Cohen-Fix and Koshland 1997; Sanchez *et al.* 1999; Wang *et al.* 2001). In addition to its role as an anaphase inhibitor, Pds1p also acts as an activator of Esp1p by promoting its nuclear localization (Jensen *et al.* 2001; Agarwal and Cohen-Fix 2002). Esp1p must have a Pds1p-independent mechanism for entering the nucleus that functions adequately at room temperature, but cells lacking Pds1p are temperature sensitive because they lack sufficient Esp1p in the nucleus to initiate anaphase (Yamamoto *et al.* 1996a; Jensen *et al.* 2001). Because Pds1p both promotes Esp1p's nuclear localization and inhibits Esp1p's activity, the cell ensures that any Esp1p that colocalizes with its nuclear substrate remains inactive until Pds1p is degraded.

The cyclin-dependent kinase (CDK), Cdc28p, is a critical regulator of cell cycle progression in budding yeast. Cdc28p pairs with at least nine different cyclins during the cell cycle (six Clbs and three Clns; reviewed in Men-

[1]*Corresponding author:* NIH/NIDDK/LMCB, 8 Center Dr., Bldg. 8, Rm. 319, Bethesda, MD 20892-0840. E-mail: ornacf@helix.nih.gov

denhall and Hodge 1998). The type of cyclin associated with Cdc28p, and perhaps the absolute level of kinase activity, determine which Cdc28p substrates get phosphorylated and to what extent. Among Cdc28p's targets are other regulatory proteins that are responsible for cell cycle processes such as the metaphase-to-anaphase transition or the exit from mitosis. Because the mechanisms controlling different aspects of cell cycle progression are highly interconnected, disruption of one part of the system may have adverse effects on later events.

Not surprisingly, Cdc28p/cyclin activity is highly regulated. Cdc28p/cyclin activity is inhibited by a number of mechanisms including phosphorylation, cyclin degradation, and binding of inhibitory proteins. Inhibitory phosphorylation of Cdc28p is carried out by Swe1p. Swe1p is the budding yeast homolog of Wee1, a kinase found in fission yeast and higher eukaryotes, which phosphorylates and inhibits mitotic Cdk/cyclin at the G_2/M transition. Unlike in other organisms, the timing of mitotic entry during normal cell cycles in budding yeast is not regulated by inhibitory phosphorylation of the mitotic CDK (Amon *et al.* 1992; Sorger and Murray 1992); nonetheless, the biochemical functions of Wee1 and Swe1p appear to be conserved. Swe1p is specific for Cdc28p/Clb complexes and probably phosphorylates Cdc28p/Clb during normal growth because mutants that cannot remove the Swe1p-dependent phosphorylation from Cdc28p show a G_2/M delay that is eliminated when *SWE1* is deleted (Booher *et al.* 1993). The degradation of the mitotic cyclins (*e.g.*, Clb2) is mediated primarily by the APC/C that is associated with Cdh1p, a Cdc20p homolog (Schwab *et al.* 2001). Cdh1p, like Cdc20p, functions as a substrate specificity factor for the APC/C (Schwab *et al.* 1997; Visintin *et al.* 1997). In addition to the mitotic Clbs, APC/C^{Cdh1p} directs the ubiquitination of a number of substrates late in mitosis and in G_1, including the septin-associated kinase Hsl1p (Barral *et al.* 1999; Burton and Solomon 2000), the spindle motor Cin8p (Hildebrandt and Hoyt 2001), and the spindle-associated protein Ase1p (Juang *et al.* 1997; Visintin *et al.* 1997). By targeting mitotic cyclins for degradation, APC/C^{Cdh1p} inactivates Cdc28p/Clbs, a prerequisite for mitotic exit. However, Cdh1p is not essential because mitotic Cdc28p/Clb activity in G_1 is also squelched by the binding of Sic1p, a CDK inhibitor. The requirement for either Cdh1p or Sic1p is underscored by the fact that although neither protein is essential, the *cdh1*Δ *sic1*Δ double mutant is inviable.

Following mitosis, both APC/C^{Cdh1p} and Sic1p continue to function as CDK inhibitors throughout G_1 of the next cell cycle (Huang *et al.* 2001). Sic1p activity during G_1 is important for DNA replication (Lengronne and Schwob 2002). Assembly of prereplication complexes on replication origins, which normally occurs during G_1, is inhibited by Cdc28p/Clb activity (Dahmann *et al.* 1995). In *sic1*Δ mutants, Cdc28p/Clb5,6p activity during G_1 is too high, and some origins are never primed for replication (Lengronne and Schwob 2002). Consequently, DNA replication is not completed in a timely manner, and some cells attempt to segregate incompletely replicated chromosomes, resulting in extensive chromosome loss. In this report we present evidence that, like Sic1p, Cdh1p is also important for more than mitotic exit. However, Cdh1p activity at the end of mitosis is distinct from that of Sic1p and is required to ensure high-fidelity chromosome segregation during mitosis of the next cell cycle.

MATERIALS AND METHODS

Yeast strains: The genotypes of the strains used in this work are listed in Table 1. Strains are derived from the W303 background except where indicated. To create strain KR3011, a *PDS1* disruption cassette was cut out of pAY55 (Yamamoto *et al.* 1996a) with *Apa*I and *Ksp*I and transformed into a *bar1*Δ version of ymw2 (gift of M. Solomon, Yale University; originally from M. Walberg and R. Davis, Stanford University). Sources of other mutant alleles used were: *bub2::URA3* (from strain KH128, gift of S. Biggins), *mad2::URA3* (from strain KH141, gift of S. Biggins), *cdh1::HIS3* [from strain 1120, gift of A. Amon, Massachusetts Institute of Technology (MIT)], *sic1::HIS3* (from strain 708, gift of A. Amon, MIT), *rad9::HIS3* (SE1, gift of S. Elledge, Baylor College of Medicine), and *mrc1::S.p. his5+* (SE2, gift of S. Elledge, Baylor College of Medicine). The sources of the chromsome III fragment (Spencer *et al.* 1990) were JCY149 and JCY150, both of which were gifts of J. Campbell (National Institutes of Health) and were made on the basis of the strategy described in Spencer *et al.* (1990). The source of the *tetO::URA3* and *GFP-tetR::LEU2* constructs was strain 6752, a gift of K. Nasmyth, Vienna.

The *cdh1::kan* (used for all *cdh1::kan* strains except 3124), *pds1::kan*, and *ade3::kan* alleles were created by PCR-based one-step gene disruption (Longtine *et al.* 1998). PCR was done with plasmid pFA6a-kanMX6 as template, forward primers that consisted of 50 bases 5′ of the coding region of the gene to be disrupted followed by F_1, and reverse primers that consisted of 50 bases 3′ of the coding region of the gene to be disrupted followed by a modified version of R1 (5′-CGA TGAATTCGAGCTCGTTT-3′). The sequences of the primers were as follows: KRO162 (cdh1-forward), 5′-CTCCGATTTTT GTCACCCTTCCTTCTAGTCTTCATCCTAAATTTAGTTGC CGGATCCCCGGGTTAATTAA-3′; KRO163 (cdh1-reverse), 5′-TTTTTTTTACAGAATTTTTGAGATGATATTACTACTAT GAAAACCCTTTACGATGAATTCGAGCTCGTTT-3′; KRO171 (pds1-forward), 5′-TTACACTTCTGCGGTACCAAGCTAGAT TAAGTGCTAGATAATAAACCTTTCGGATCCCCGGGTT AATTAA-3′; KRO172 (pds1-reverse), 5′-TATCTGTATATAC GTGTATATATGTTGTGTGTATGTGAATGAGCAGTGGAT CGATGAATTCGAGCTCGTTT-3′; KRO197 (ade3-forward), 5′-TGAGACCAGGTAACGAGACGAACACAACTTTACAAGT CAAATAAGAAATCCGGATCCCCGGGTTAATTAA-3′; KRO198 (ade3-reverse), 5′-AAAAAAACTTTTGCATTTGTCTTTATTA AATTCTATATAATTAAGTTGTCCGATGAATTCGAGCTC GTTT-3′.

swe1::kan with ~400 bases of flanking sequence 5′ and 3′ was amplified by PCR from a *swe1::kan* strain (S228c background; American Type Culture Collection, Manassas, VA) using primers KRO140 (swe1-forward) 5′-GTGGGAGATAGGGGGCTA TTCG-3′ and KRO141 (swe1-reverse) 5′-GAACTTTTGGTG GTCCAGCGTGG-3′ and transformed into W303.

The *mad2::ura3::HIS3* allele was created using the marker

TABLE 1

Strain list

Strain	Genotype	Source or reference
ymw2	*W303*[a] *MAT***a** *ade3*	M. Solomon
KR3011	W303 *MAT***a** *ade3 pds1::LEU2*	This work
SL189	W303 *MAT***a** *ade3 pds1::LEU2 cdh1-189*	This work
KH128	W303 *MAT***a** *bub2::URA3*	S. Biggins
KH141	W303 *MAT***a** *mad2::URA3*	S. Biggins
1120	W303 *MAT***a** *can1-100 GAL psi*$^+$ *cdh1::HIS3 PDS1-HA::LEU2*	A. Amon
708	W303 *MAT***a** *can1-100 GAL psi*$^+$ *sic1::HIS3*	A. Amon
JCY149	W303 *MAT*α *CFIII*[b]	J. Campbell
JCY150	W303 *MAT***a** *CFIII*[b]	J. Campbell
6752	W303 *MAT*α *can1-100 scc1 tetO::URA3 GFP-tetR::LEU2*	K. Nasmyth
SE1	W303 *MAT***a** *rad9::HIS3*	S. Elledge
SE2	W303 *MAT***a** *mrc1::S.p. his5+*	S. Elledge
4521-001	*MAT*α *leu2 ade2 ade3 can1 sap3 ura1 his7 gal1* (A364a background)	D. Koshland
4525-061	*MAT*α *leu2 ade2 ade3 can1 sap3 ura1 his7 gal1 cdc6-1* (A364a background)	D. Koshland
KR3038-6D	W303 *MAT***a** *cdh1::HIS3*	This work
KR3044-1B	W303 *MAT***a** *cdh1::kan*	This work
KR3044-3A	W303 *MAT*α *cdh1:kan pds1::LEU2*	This work
KR3054-8D	W303 *MAT***a** *cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3055-10A	W303 *MAT***a** *tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3055-17B	W303 *MAT*α *cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3056-1A	W303 *MAT***a** *pds1::kan cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3056-1C	W303 *MAT***a** *cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3056-8A	W303 *MAT***a** *tetO-URA3::ura3 GFP-tetR-LEU2::leu*	This work
KR3056-12C	W303 *MAT***a** *pds1::kan cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3056-12D	W303 *MAT*α *pds1::kan cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3057-7C	W303 *MAT***a** *pds1::kan tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3057-18A	W303 *MAT***a** *pds1::kan tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3062-2B	W303 *MAT*α *mad2::ura3::HIS3 CFIII*[b]	This work
KR3062-7C	W303 *MAT*α *mad2::ura3::HIS3 CFIII*[b]	This work
KR3066-3B	W303 *MAT***a** *cdh1::HIS3 mad2::URA3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3066-3D	W303 *MAT***a** *tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3066-6A	W303 *MAT***a** *cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3066-7B	W303 *MAT***a** *cdh1::HIS3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3066-7D	W303 *MAT***a** *cdh1::HIS3 mad2::URA3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3066-8C	W303 *MAT***a** *tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3067-4B	W303 *MAT***a** *mad2::URA3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3067-6B	W303 *MAT***a** *cdh1::HIS3 mad2::URA3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3068-3C	W303 *MAT***a** *mad2::URA3 tetO-URA3::ura3 GFP-tetR-LEU2::leu2*	This work
KR3071-2B	W303 *MAT***a** *cdh1::HIS3 bub2::URA3*	This work
KR3072-3D	W303 *MAT***a** *mad2::URA3 cdh1::HIS3*	This work
KR3074-1B	W303 *MAT***a**	This work
KR3077-4A	W303 *MAT*α *mad2::ura3::HIS3 cdh1::kan CFIII*[b]	This work
KR3077-10B	W303 *MAT*α *cdh1::kan CFIII*[b]	This work
KR3077-11A	W303 *MAT*α *mad2::ura3::HIS3 cdh1::kan CFIII*[b]	This work
KR3078-2A	W303 *MAT*α *CFIII*[b]	This work
KR3078-2B	W303 *MAT*α *CFIII*[b]	This work
KR3078-5B	W303 *MAT*α *cdh1::kan CFIII*[b]	This work
KR3085-1C	W303 *MAT*α *ade3::kan*	This work
KR3085-2A	W303 *MAT***a** *ade3::kan mad2::URA3*	This work
KR3085-6C	W303 *MAT*α *ade3::kan cdh1::HIS3*	This work
KR3085-8C	W303 *MAT***a** *ade3::kan*	This work
KR3085-14A	W303 *MAT***a** *ade3::kan cdh1::HIS3*	This work
KR3085-15D	W303 *MAT*α *ade3::kan mad2::URA3*	This work
KR3085-18A	W303 *MAT*α *ade3::kan mad2::URA3 cdh1::HIS3*	This work
KR3085-19D	W303 *MAT***a** *ade3::kan mad2::URA3 cdh1::HIS3*	This work
KR3089-1C	W303 *MAT*α *sic1::HIS3*	This work
KR3089-1D	W303 *MAT*α	This work

(*continued*)

TABLE 1

(Continued)

Strain	Genotype	Source or reference
KR3089-4D	W303 *MAT*α *pds1::LEU2 sic1::HIS3*	This work
KR3089-6C	W303 *MAT*α *pds1::LEU2*	This work
KR3091-3D	W303 *MAT***a** *cdh1::kan rad9::HIS3*	This work
KR3093-10A	W303 *MAT***a** *cdh1::kan mrc1::S.p. his5+*	This work
KR3098-2A	W303 *MAT*α *mad2::URA3 cdh1::HIS3*	This work
KR3098-6B	W303 *MAT*α *mad2::URA3 swe1::kan*	This work
KR3098-12B	W303 *MAT*α *cdh1::HIS3 swe1::kan*	This work
KR3099-3B	W303 *MAT*α *mad2::URA3 cdh1::HIS3 swe1::kan*	This work
KR3118	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 hxt13::URA3* (S288c background)	This work
KR3123	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 hxt13::URA3 sic1::kan* (S288c background)	This work
KR3124	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 hxt13::URA3 cdh1::kan* (S288c background)	This work

[a] W303: *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1.*
[b] CFIII refers to the chromosome III fragment described in Spencer *et al.* (1990).

swap plasmid method as described in Cross (1997). Plasmid pUH7 was digested with *Sma*I to release *HIS3* flanked by *URA3* sequences. The fragment was transformed into a *mad2::URA3* strain and his^+ ura^- transformants were isolated. The above strains were crossed with each other and with other appropriately marked W303 strains to create the strains in Table 1 numbered KR3038–KR3099-3B.

hxt13::URA3 was constucted by PCR using pRS306 (Sikorski and Hieter 1989) as a template and primers consisting of 50 bases 5′ and 3′ to *HXT13* followed by sequences that anneal to the regions flanking the *URA3* gene in pRS306 [KRO230 (hxt13-forward) 5′-CACGTAAGGCATAACAATCAAAAAAAG AAAAAAGAAACAAAAGTTAAACCGCATCAGAGCAGATT GTACTG-3′ and KRO231 (hxt13-reverse) 5′-AACTATAATA TACAATGTTGCCTATCAAGACAAACATATGCACTCTATG ACTCCTTACGCATCTGTGCGG-3′]. The PCR product was tranformed into BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*; S288c background; Yeast Consortium, ResGen, Invitrogen, Huntsville, AL) to create strain 3118. *sic1::kan* and *cdh1::kan* (strain 3124) with 400 bases of flanking sequence 5′ and 3′ were amplified from the *sic1::kan* and *cdh1::kan* strains from the Saccharomyces Genome Deletion Project *MAT***a** Collection (Yeast Consortium; ResGen, Invitrogen) using the following primers: KRO235 (sic1-forward), 5′-GGCCAACTCTTGTT GTAGTTG-3′; KRO195 (sic1-reverse), 5′-GTCACTTCTAGCA AATTTGG-3′; KRO236 (cdh1-forward 2), 5′-GTCTCCACCA TAACCATAGAAG-3′; and KRO164 (cdh1-reverse 2), 5′-GAC GCCTGTAATATGTCATG-3′. The PCR products were transformed into strain 3118 to create strains 3123 and 3124.

Media: Liquid yeast culture media was prepared as described in Ausubel *et al.* (1995). Amino acid dropout powders for synthetic media were purchased from Bio101 (Carlsbad, CA). YP + galactose solid media were prepared like YP + dextrose (Ausubel *et al.* 1995) except that 2% galactose was substituted for dextrose. 5-Fluoroanthranilic acid (FAA) solid media was prepared as described in Toyn *et al.* (2000) with FAA purchased from Aldrich Chemical (Milwaukee). Other solid yeast media and Luria broth (LB) + ampicillin plates were purchased from KD Medical (Columbia, MD). *Escherichia coli* minimal medium + leucine and tryptophan contained: 1.5% agar, 1× M9 salts (6 μg/ml Na_2HPO_4, 3 μg/ml KH_2PO_4, 0.5 μg/ml NaCl, and 1 μg/ml NH_4Cl), 1 mm magnesium sulfate, 1 μm $FeCl_3$, 1 μg/ml thiamine, 0.5% dextrose, and 26 μg/ml each tryptophan and leucine.

Plasmids: *PDS1* was cut from pOC20 [*CEN/URA3/PDS1*, with the *PDS1* gene inserted between the *Eco*RI and *Bam*HI sites of plasmid pRS316 (Sikorski and Hieter 1989)] with *Bam*HI and *Eco*RI and ligated into the corresponding sites in pRS314 (CEN/TRP1; Sikorski and Hieter 1989) to create pKR201. ADE3 (coding region plus 500 bases on each side) was amplified by PCR from pDK255 (gift of D. Koshland) with primers that introduced a 5′ *Sac*I site (KRO105) and a 3′ *Not*I site (KRO106). Primer sequences were: KRO105, 5′-GGGTAT GAGCTCTACGTGAGCTAAAGCACAGATTG-3′; and KRO106, 5′-GGATAAGCGGCCGCGTAGTCCAATACCGTTTTTG-3′. The PCR product was digested with *Sac*I and *Not*I and ligated into the corresponding sites of pKR201 to create pKR204. Plamids 189-26 (*CDH1*) and 189-20 (*SWE1*) were isolated from a *CEN/URA3* genomic library (Rose *et al.* 1987). The genomic insert in plasmid 189-26 corresponds to chromosome VII bases 485,079–496,384. The genomic insert in plasmid 189-20 corresponds to chromosome X bases 74,516–80,656. To create pKR252 (*CEN/TRP1/CDH1*), plasmid 189-26 was cut with *Eco*RI, and the resulting 6.7-kb fragment including *CDH1* was ligated into pRS314 (CEN/TRP1; Sikorski and Hieter 1989). pJM1091 (gift of J. Harrison, Duke University) consists of the *SWE1* open reading frame with ~900 bases of promoter sequence and 10–12 C-terminal MYC tags in pRS316 (*CEN/URA3*; Sikorski and Hieter 1989). AD10 [*SIC1* in pRS425 (2μ/*LEU2*; Sikorski and Hieter 1989)] was a gift of F. Cross, Rockefeller University. Plasmids pDK243, pDK368-1, and pDK368-7 (gifts of E. Hogan and D. Koshland, Carnegie Institute) were described in Hogan and Koshland (1992).

Synthetic lethal screening: The synthetic lethal screen was based on the sectoring strategy of Bender and Pringle (1991). Strain KR3011 (*pds1*Δ *ade2 ade3 trp1*) with the nonessential plasmid pKR204 (*CEN/TRP1/ADE3/PDS1*) was grown overnight in 25 ml synthetic complete media lacking tryptophan. Cells were washed with water and resuspended in 25 ml 0.1 m sodium phosphate buffer, pH 7.0. Threemilliliter aliquots of cells were incubated in either 100 μl (27 mm) or 150 μl (40 mm) of methanesulfonic acid ethyl ester (EMS; Sigma, St. Louis) for 90 min. Viability after this treatment ranged from 10 to 30%. The EMS was inactivated by washing twice with 3 ml 5% sodium thiosulfate. Cells were resuspended in media, plated on YP + dextrose after the appropriate dilution to get ~300 colonies per plate, and incubated at 23°. A total of 18,000 colonies were screened. Colonies that failed to sector (*i.e.*, completely red colonies) were restreaked and then tested for sensitivity to FAA that selects

against the *TRP1* plasmid, pKR204. Nonsectoring, FAA-sensitive strains were kept for further analysis.

Cloning of SL189: Synthetic lethal candidate strain SL189 with pKR204 was transformed with a *CEN/URA3* yeast genomic library (Rose *et al.* 1987). Transformants were selected on synthetic complete media lacking uracil and then replica plated onto FAA to select against pKR204. Thirty-eight FAA-resistant colonies were isolated out of ~14,000 transformants. Library plasmids were recovered from yeast by the yeast-boiling DNA miniprep procedure (Robzyk and Kassir 1992) and transformed into *E. coli*. Plasmids were sequenced using primers RO91 5′-GCTTTGGCCGCCGCCCAGTCCTGCTGCC and RO92 5′-CATCGGTGATGTCGGCGATATAGGCGCC that flank the genomic DNA insert.

Gap repair: The *CDH1*-containing library plasmid, 189-37 (genomic insert equals chromosome VII bases 483,481–496,380), was digested with *Pvu*II to remove 7.9 kb of the genomic insert including *CDH1*. The backbone was gel purified and religated to create pKR217. The *SWE1*-containing library plasmid, 189-10 (genomic insert equals chromosome X bases 68,067–80,656), was digested with *Afl*II and *Sna*BI to remove 10.1 kb of the genomic insert including *SWE1*. (*Sna*BI cut the insert into two pieces so it would not comigrate with the backbone on a gel.) The backbone was gel purified and religated to create pKR216. Strain SL189 (carrying pKR204) was transformed with pKR217 that had been linearized with *Pvu*II or pKR216 that had been linearized with *Afl*II. Plasmids (a mixture of pKR204 and pKR216 or pKR217 derivatives) were isolated from ura$^+$ transformants by the yeast boiling DNA miniprep procedure (Robzyk and Kassir 1992) and transformed into *E. coli* strain MC1066 [*galU galK strAr hsdR*$^-$ δ*(lacIPOZYA)X74 trpC9830 leuB6 pyrF74::Tn5(km^r)*] in which it is possible to select for the yeast *URA3* gene. Transformants were plated on LB + ampicillin and then replica plated onto minimal media + tryptophan and leucine to select for *URA3*-containing plasmids. Restriction digests were performed to determine whether the gap repair was successful.

Measurement of chromosome fragment, plasmid, and chromosome loss: *ade2* mutant cells were transformed with a nonessential fragment of chromosome III that carries the *ADE2* and *URA3* genes (Spencer *et al.* 1990) (Table 2). Loss of the fragment gave rise to red sectors in an otherwise white colony. Strains without the *SIC1* plasmid were grown overnight in synthetic complete medium lacking uracil to select for the chromosome fragment. Strains carrying the *SIC1* plasmid were grown overnight in synthetic complete medium lacking uracil and leucine to select for the plasmid as well. Cultures were diluted to a density corresponding to ~300 colonies per plate and plated on YP + dextrose. Cells that lost the chromosome fragment during the first division on the plate gave rise to half-sectored colonies whereas those that lost the fragment before plating formed completely red colonies. The percentage loss rate per cell division was calculated using the formula: 100 × (half-sectored colonies)/(total colonies − red colonies). Between 3000 and 6000 colonies without the *SIC1* plasmid and between 1000 and 2000 colonies with the *SIC1* plasmid were scored for each genotype.

pDK243 and pDK368-7 plasmid loss rates (Table 6) were determined similarly except that the strains used were *ade2-ade3* double mutants and the plasmid carried the *ADE3* gene so that cells that lost the plasmid gave rise to white sectors in an otherwise red colony (Koshland *et al.* 1985). The wild-type (WT; A364a) and *cdc6-1* strain cells were grown to log phase at 23°, incubated at 36° for 3 hr, and then plated immediately. All other strains were grown at 23° and then plated. For all strains, plates were incubated at 23°. Between 400 and 1000 colonies were scored for each genotype/plasmid combination.

Missegregation of chromosome V (Tables 3 and 4) was monitored using strains that expressed a tet repressor-green fluorescent protein (GFP) fusion and carried an array of tet operators integrated at the *URA3* locus on chromosome V (Michaelis *et al.* 1997). Cells were grown to midlog phase in YP + dextrose, fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei, and observed under the microscope. Segregation was classified as normal if a single GFP dot was observed in unbudded, small-budded, or large-budded cells with a single nucleus, or if two dots were observed in postanaphase cells, one dot in each nucleus. Other patterns were scored as missegregation events. Approximately 200–400 cells were scored for each genotype.

Gross chromosomal rearrangement assay: Gross chromosomal rearrangement (GCR) assays were done as described in Myung *et al.* (2001). *URA3* was integrated into the *HXT13* locus, which is located 7.5 kb telomeric to the *CAN1* locus on the left arm of chromosome V. Between 10^9 and 5×10^9 *hxt13::URA3* cells were grown to midlog phase in YPD and plated on canavanine/fluoroorotic acid (FOA) plates (10^9 cells per 150-mm plate) to select for cells that had lost both *URA3* and *CAN1*. Dilutions of the same cultures were plated on synthetic complete medium to determine the total number of viable cells.

Microscopy: Cells were fixed for microscopy in media with 4% paraformaldehyde (Electron Microscopy Services, Fort Washington, PA) for 1 hr at 23°, washed with 1× phosphate-buffered saline (PBS), and stored at 4°. Immediately before observation, the fixed cells were sonicated gently to break up clumps, incubated in 1% Triton-X-100 for 5 min, mixed at a 1:1 ratio with Vectashield with DAPI mounting medium (Vector Laboratories, Burlingame, CA), and placed on a slide. Observations were done with a Nikon Eclipse E800 microscope with a Nikon 100× Plan Apo phase objective and filter sets for DAPI and GFP.

RESULTS

A screen for mutations that are lethal in combination with *pds1*Δ: To search for novel proteins important for cell cycle progression, we conducted a synthetic lethal screen to identify mutants that are dependent on Pds1p for viability at 23°, a temperature at which, under normal growth conditions, Pds1p is not required. We anticipated that this approach will reveal several different classes of proteins: those acting in parallel to Pds1p in promoting nuclear localization of Esp1p or factors involved in processes such as spindle assembly or DNA metabolism whose absence would render cells dependent on Pds1p's checkpoint function. Following the strategy of Bender and Pringle (1991), we deleted the chromosomal copy of *PDS1* in an *ade2 ade3 trp1* mutant strain and provided *PDS1* on a *CEN* plasmid that also carried *TRP1* and *ADE3* (pKR204). Cells that carried the plasmid were red due to the *ade2* mutation whereas cells without the plasmid were white due to their *ade2 ade3* genotype. Under nonselective conditions, cells occasionally lost the plasmid, giving rise to white sectors in an otherwise red colony. If, however, cells sustained a mutation that rendered *PDS1* essential, viability then depended on the pKR204 plasmid, resulting in solid red colonies. The *pds1*Δ *ade2 ade3 trp1*/pKR204 strain was mutagenized with EMS and strains that formed red,

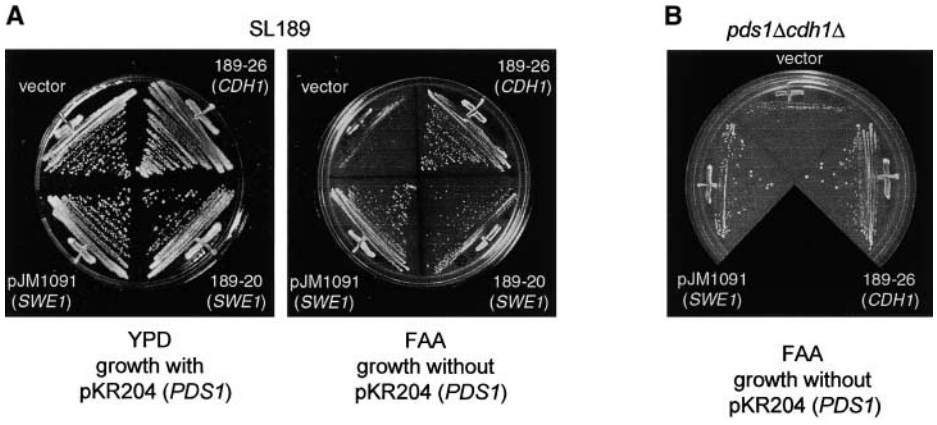

FIGURE 1.—The effect of *CDH1* and *SWE1* plasmids on the growth of *pds1 cdh1* double mutants. (A) Strain SL189 (*pds1*Δ *cdh1-189*) carrying plasmid pKR204 (*CEN/TRP1/ADE3/PDS1*) was transformed with pRS316 (*CEN/URA3*, vector), *CEN/URA3* genomic library plasmids containing *CDH1* (189-26), *SWE1* (189-20), or a *CEN/URA3* plasmid containing the *SWE1* gene alone (pJM1091). Transformants were grown at 23° on YP + dextrose (YPD; left) or FAA (right) to select for cells that have lost pKR204. (B) *pds1*Δ *cdh1*Δ cells (strain KR3044-3A) carrying plasmid pKR204 were transformed with pRS316, 189-26, or pJM1091 and grown at 23° on FAA.

nonsectoring colonies on YP + dextrose at 23° were isolated. The nonsectoring strains were then tested for FAA sensitivity. FAA is toxic to cells that have a wild-type copy of *TRP1* (TOYN *et al.* 2000), thus allowing growth only of cells that had lost the Trp1p-encoding plasmid, pKR204. Strains that required *PDS1* for viability were unable to grow without pKR204 and, consequently, were FAA sensitive. Finally, we eliminated those strains that remained FAA sensitive after transformation with a *CEN/URA3/PDS1* plasmid (for example, those that integrated pKR204 into the yeast genome). Out of 18,000 mutagenized cells, we obtained 19 synthetic lethal mutants that fell into 15 complementation groups. In this work, we describe the characterization of one of these strains, SL189, which has a mutation in the gene encoding for the APC/C activator, *CDH1* (SCHWAB *et al.* 1997; VISINTIN *et al.* 1997).

Identification of *CDH1* as the mutated gene in SL189: Strain SL189 + pKR204 (*CEN/TRP1/PDS1*) was transformed with a *CEN/URA3* yeast genomic library. Cells that obtained a library plasmid that allowed them to grow without pKR204 were selected on the basis of FAA resistance. Library plasmids that conferred FAA resistance had genomic inserts that included *PDS1*, *CDH1* (plasmid 189-26), and the CDK inhibitor *SWE1* (plasmid 189-20; Figure 1A). To determine whether the *SWE1* or *CDH1* genes were mutated in SL189, we isolated the genomic alleles of these genes from strain SL189 by gap repair of the corresponding library plasmids from which the coding regions of these genes were removed. The genomic copy of *SWE1* in SL189 appeared to be functional because the *SWE1* gap-repaired plasmid allowed SL189 to grow without the *PDS1* plasmid (data not shown). We also confirmed that a centromeric plasmid carrying the wild-type *SWE1* gene alone (pJM1091; Figure 1A) was able to suppress the synthetic lethality of SL189. Thus, *SWE1* is probably a suppressor of the *pds1*Δ synthetic lethality in SL189. The *CDH1* gap-repaired plasmid, on the other hand, could not suppress the synthetic lethality of SL189, suggesting that the mutation responsible for the synthetic lethality had been transferred to the *CDH1* plasmid. We sequenced the gap-repaired *CDH1* allele and found that it had a C-to-T mutation at base 460 (out of 1701 bases) that changed the codon for Arg 154 to a STOP codon (this allele was named *cdh1-189).* To test directly whether *cdh1*Δ is synthetically lethal with *pds1*Δ, the meiotic products of a *pds1*Δ*/PDS1 cdh1*Δ*/CDH1* heterozygous diploid were analyzed, and no viable double-mutant spores were identified. When the heterozygous diploid was transformed with a *PDS1* plasmid before sporulation, viable double-mutant spores were obtained, but they all required the *PDS1* plasmid for viability. *pds1*Δ *cdh1*Δ cells transformed with plasmids expressing either *CDH1* (plasmid 189-26) or *SWE1* (pJM1091) were able to grow in the absence of the *PDS1* plasmid (Figure 1B). On the basis of this evidence, we conclude that *pds1*Δ and *cdh1*Δ are synthetically lethal and that the nonsense mutation in *cdh1-189* is likely to be responsible for the synthetic lethality with *pds1*Δ in SL189.

Mutations in the spindle assembly checkpoint are deleterious to *cdh1*Δ mutants: We next investigated which function(s) of Pds1p was required for viability in *cdh1*Δ mutants. Overexpression of *ESP1* from a galactose-inducible promoter did not rescue the *pds1*Δ *cdh1*Δ mutant, suggesting that Pds1p's role in Esp1p activation was unlikely to be relevant to the *pds1*Δ *cdh1*Δ synthetic lethality (data not shown). Pds1p is an essential part of the spindle assembly checkpoint pathway (GARDNER and BURKE 2000) and also assists in arresting the cell cycle after DNA damage (YAMAMOTO *et al.* 1996b; COHEN-FIX and KOSHLAND 1997; GARDNER *et al.* 1999). If Pds1p's checkpoint function(s) was important for the viability of *cdh1*Δ mutants, we would expect to see genetic interactions between *CDH1* and other checkpoint proteins. Thus, we created mutants that lacked Cdh1p and a component of each of four cellular checkpoint pathways: (1) the spindle assembly checkpoint (Mad2p; Figure 2, A and C), (2) the DNA damage checkpoint (Rad9p; Figure 2, B and C), (3) the DNA replication

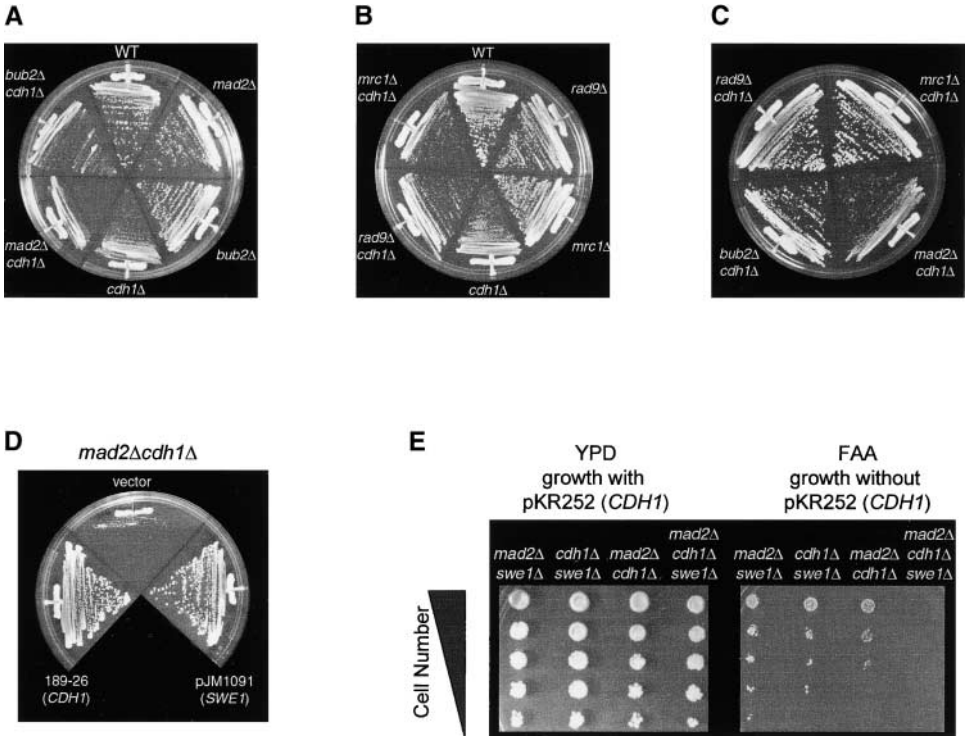

FIGURE 2.—Genetic interactions between *cdh1*Δ and checkpoint mutants. (A) Wild-type (strain KR3074-1B, WT), *mad2*Δ (strain KH141), *bub2*Δ (strain KH128), *cdh1*Δ (strain KR3038-6D), *mad2*Δ *cdh1*Δ (strain KR3072-3D), and *bub2*Δ *cdh1*Δ (strain KR3071-2B) cells were grown on YP + dextrose at 23°. (B) Wild-type (strain KR3074-1B, WT), *rad9*Δ (strain SE1), *mrc1*Δ (strain SE2), *cdh1*Δ (strain KR3044-1B), *rad9*Δ *cdh1*Δ (strain KR3091-3D), and *mrc1*Δ *cdh1*Δ (strain KR3093-10A) cells were grown on YP + dextrose at 23°. (C) The double-mutant combinations *rad9*Δ *cdh1*Δ (strain KR3091-3D), *mrc1*Δ *cdh1*Δ (strain KR3093-10A), *mad2*Δ *cdh1*Δ (strain KR3072-3D), and *bub2*Δ *cdh1*Δ (strain KR3071-2B) were grown on YP + dextrose at 23°. (D) *mad2*Δ *cdh1*Δ cells (strain KR3074-3B) were transformed with pRS316 (*CEN/URA3*, vector), a *CEN/URA3* genomic library plasmid containing *CDH1* (189-26), or a *CEN/URA3* plasmid expressing *SWE1* alone (pJM1091). Transformants were grown on synthetic medium lacking uracil at 23°. (E) *mad2*Δ *swe1*Δ (strain KR3098-6B), *cdh1*Δ *swe1*Δ (strain KR3098-12B), *mad2*Δ *cdh1*Δ (strain KR3098-2A), and *mad2*Δ *cdh1*Δ *swe1*Δ (strain KR3099-3B) cells all carrying a *CEN/TRP1/CDH1* plasmid (pKR252) were spotted onto YP + dextrose plates (left) or FAA plates (right) at 23° to determine whether they could grow in the absence of pKR252.

checkpoint (Mrc1p; ALCASABAS *et al.* 2001; TANAKA and RUSSELL 2001; Figure 2, B and C), and (4) the spindle-positioning checkpoint (Bub2p; HOYT 2000; Figure 2, A and C), although Pds1p has so far not been shown to be involved in this checkpoint. We found that *cdh1*Δ single mutants grew significantly more slowly than wild type or any of the other single mutants (Figure 2, A and B). *mad2*Δ *cdh1*Δ double mutants, while viable, were much slower growing than the *cdh1*Δ single mutant (Figure 2A). This interaction was specific to the spindle assembly checkpoint as *rad9*Δ *cdh1*Δ and *mrc1*Δ *cdh1*Δ double mutants grew as well as *cdh1*Δ single mutants (Figure 2B), and *bub2*Δ *cdh1*Δ double mutants were, at most, slightly slower growing (Figure 2A).

The spindle assembly checkpoint pathway delays the metaphase-to-anaphase transition until all chromosomes are properly attached to the spindle. Therefore, mutants with compromised spindle or kinetochore function would be expected to exhibit a checkpoint-dependent G_2/M delay, which would be manifested by an abnormally high proportion of G_2/M cells in an asynchronously growing culture. The fraction of G_2/M cells in an asynchronously growing culture of the *cdh1*Δ single mutant was similar to wild type (Figure 3). We also saw no significant differences among wild-type, *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ strains when we timed the interval between bud emergence and nuclear division in single cells, although we would not have been able to detect delays of <15 min (data not shown). It is possible that *cdh1*Δ cells undergo a G_2/M delay that is too brief to dramatically affect the overall cell cycle distribution of cells in an asynchronous culture but is nonetheless important for cell survival (see below).

Like *pds1*Δ *cdh1*Δ mutants, *mad2*Δ *cdh1*Δ cells were sensitive to *SWE1* levels. *mad2*Δ *cdh1*Δ strains grew better when extra *SWE1* was provided on a centromeric plasmid (pJM1091, Figure 2D), and conversely, *mad2*Δ *cdh1*Δ *swe1*Δ triple mutants were inviable (Figure 2E). These results suggest that the growth defects in the *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ strains may have the same underlying cause. Because *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ strains are affected by the level of Swe1p, an inhibitor of the cell cycle kinase, Cdc28p/Clb (BOOHER *et al.* 1993), and because Clb cyclins are known targets of Cdh1p (SCHWAB *et al.* 1997, 2001), the mutant phenotypes of these strains may be related to abnormal regulation of Cdc28p/Clb activity.

***mad2*Δ *cdh1*Δ strains exhibit extensive chromosome loss:** The spindle assembly checkpoint pathway is necessary for high-fidelity chromosome transmission in cells in which the spindle or kinetochores are compromised in some way. The poor growth of *mad2*Δ *cdh1*Δ raised the possibility that *cdh1*Δ mutant cells had spindle/kinetochore defects that, in the absence of the spindle checkpoint, led to chromosome missegregation followed by cell lethality. To test this idea, we compared

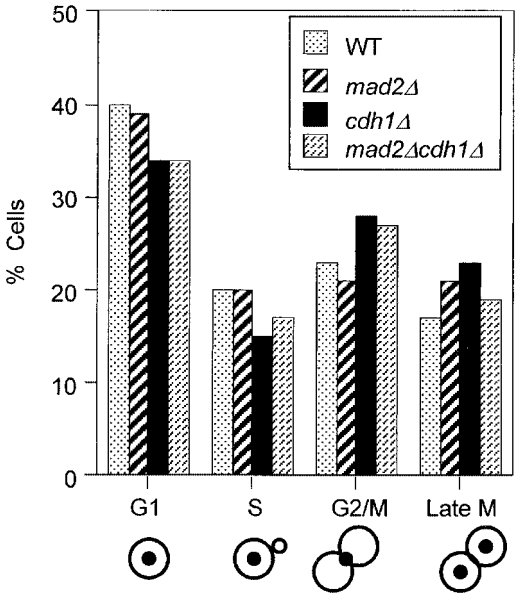

FIGURE 3.—The cell cycle distribution of asynchronous populations of wild-type, *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ cells. Wild-type (WT; strains KR3066-8C and KR3066-3D), *mad2*Δ (strains KR3067-4B and KR3068-3C), *cdh1*Δ (strains KR3066-6A and KR3066-7B), and *mad2*Δ *cdh1*Δ (strains KR3066-3B, KR3066-6B, and KR3066-7D) cells were grown to midlog phase in YP + dextrose, fixed, stained with DAPI to visualize nuclei, and examined under the microscope. Cells were classified as unbudded (G_1), small budded (S), large budded with a single nucleus (G_2/M), or large budded with nuclei in mother and bud (late M). Results are averages of two independent experiments for each strain.

the loss rate of a nonessential fragment of chromosome III (SPENCER *et al.* 1990) in wild-type, *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ strains (Table 2, left column). The fragment is transmitted during mitosis of wild-type cells nearly as well as native chromosomes and it carries the *ade2* suppressor, *SUP11*, allowing its presence to be monitored in a colony color assay (SPENCER *et al.* 1990). We calculated the percentage of half-sectored colonies, which reflects fragment loss during the first mitotic division on the plate. Of 6000 wild-type colonies examined, none had half-sectors, implying that the loss rate was no greater than 0.02% per division. This result is in agreement with the published value of 0.017% per division (SPENCER *et al.* 1990). The *mad2*Δ strain had a loss rate of 0.4% (at least 20-fold greater than that of wild type), which is also consistent with published data (WARREN *et al.* 2002). In the *cdh1*Δ strain chromosome fragment loss was elevated at least 180-fold over wild type; in the *mad2*Δ *cdh1*Δ double mutant, fragment loss was at least 400-fold greater than that of wild type. Deletion of *CDH1* alone substantially reduces the fidelity of chromosome transmission, suggesting that some of the damage caused by this mutation is not recognized by the spindle checkpoint; however, the checkpoint is exerting a protective effect because the loss rate is significantly higher in the *cdh1*Δ *mad2*Δ double mutant.

TABLE 2

Loss of chromosome III fragment

Strain	% chromosome fragment loss	
	−	+ *SIC1*
WT	<0.02	<0.07
*mad2*Δ	0.4 ± 0.1	0.7 ± 0.2
*cdh1*Δ	3.6 ± 0.6	0.7 ± 0.4
*mad2*Δ *cdh1*Δ	8.3 ± 4.4	4.6 ± 1.2

The percentage of loss rate per cell division of a nonessential fragment of chromosome III was determined for wild-type (WT, strains KR3078-2A and KR3078-2B), *mad2*Δ (strains KR3062-2B and KR3062-7C), *cdh1*Δ (strains KR3077-10B and KR3078-5B), and *mad2*Δ *cdh1*Δ (strains KR3077-11A and KR3077-4A) cells without (−) or with (+ *SIC1*) a 2μ *SIC1* plasmid as described in MATERIALS AND METHODS. For wild type, no half-sectored colonies were observed so an upper limit on the rate of first division fragment loss was calculated using the formula 100 × 1/total colonies scored. For the other strains, the average ± standard deviation of two or four independent experiments is shown.

Chromosome transmission was also monitored by an assay in which chromosome V is visualized by expression of a GFP-tagged tet repressor that binds to an array of tet operators integrated into the *URA3* locus of chromosome V (MICHAELIS *et al.* 1997; Table 3). In a wild-type population, preanaphase cells exhibit a single GFP spot, while postanaphase cells exhibit two GFP spots, one in the mother nucleus and one in the bud nucleus. Other patterns can arise from chromosome transmission errors. When we examined the distribution of GFP spots in wild-type, *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ strains, our results agreed well with the results of the chromosome fragment loss assay: wild-type cells showed no evidence of errors, *mad2*Δ and *cdh1*Δ cells had a moderate level (1%), and *mad2*Δ *cdh1*Δ cells had a greatly elevated level (6.1%). Importantly, all of the abnormal patterns counted were consistent with missegregation events in which both sister chromatids segregated to the same cell (*e.g.*, unbudded cells with more than one GFP spot or postanaphase cells with two spots in the same nucleus). If the other 15 chromosomes in *mad2*Δ *cdh1*Δ are missegregated as often as chromosome V (~6%), then the fraction of cells in the *mad2*Δ *cdh1*Δ culture that have the correct complement of chromosomes is only $(1 - 0.06)^{16} = 0.37$. Assuming that most missegregation events resulting in chromosome loss are lethal, this frequency of chromosome missegregation could easily account for the slow growth of *mad2*Δ *cdh1*Δ strains. Finally, as mentioned earlier, *mad2*Δ *cdh1*Δ cells do not accumulate in any particular phase of the cell cycle (Figure 3); instead, it is likely that they stop growing whenever a critical protein encoded by the chromosome(s) they have lost becomes limiting.

Chromosome segregation in *pds1*Δ *cdh1*Δ: To study the terminal phenotype of the *pds1*Δ *cdh1*Δ strain, we

TABLE 3

Missegregation of chromosome V

Strain	% missegregation of chromosome V
WT	0
*mad2*Δ	1.0
*cdh1*Δ	1.0
*mad2*Δ *cdh1*Δ	6.1

Wild-type (WT, strains KR3066-8C and KR3066-3D), *mad2*Δ (strains KR3067-4B and KR3068-3C), *cdh1*Δ (strains KR3066-6A and KR3066-7B), and *mad2*Δ *cdh1*Δ (strains KR3066-3B, KR3066-6B, and KR3066-7D) cells were grown in YP + dextrose and scored for missegregation of GFP-tagged chromosome V as described in MATERIALS AND METHODS and text.

exploited the fact that this strain, while dead in YP + dextrose, does grow, albeit poorly, in YP + galactose (F. CROSS, personal communication; Figure 4A). We do not have an explanation for this phenomenon. We considered the possibility that the *pds1*Δ *cdh1*Δ strain benefited from progressing through the cell cycle more slowly, a consequence of using a suboptimal carbon source like galactose. However, *pds1*Δ *cdh1*Δ did not grow in dextrose synthetic complete medium, in which the doubling time of wild-type cells is comparable to that in YP + galactose (data not shown). There is accumulating evidence that a carbon source affects the expression of many genes, including some cell cycle regulatory genes (CROSS *et al.* 2002). It is plausible that the expression of factors involved in spindle assembly or cell cycle control may be altered in the presence of galactose in a way that is helpful to *pds1*Δ *cdh1*Δ cells. When *pds1*Δ *cdh1*Δ cells growing in YP + galactose were switched to YP + dextrose, they lost viability over a period of days (Figure 4B). We examined these cells for chromosome segregation defects using the tetO/GFP-tetR system described above (Table 4). All wild-type cells examined showed normal segregation of chromosome V in both dextrose- and galactose-containing media. In galactose, ~70% of *pds1*Δ *cdh1*Δ cells were viable, and 5.6% had missegregated chromosome V. After 48 hr in dextrose, viability had dropped to 6% and >20% of cells had abnormal GFP patterns. In *pds1*Δ and *cdh1*Δ single-mutant cells, missegregation levels were higher in dextrose than in galactose (2.1 *vs.* 0% for *pds1*Δ cells and 2.1 *vs.* 1.0% for *cdh1*Δ cells), but in all cases, missegregation events were far less frequent in the single mutants than in the double mutant.

Finally, the cell cycle distributions of *pds1*Δ *cdh1*Δ strains in both galactose and dextrose were similar overall to those of wild type and the two single mutants (Figure 4, C and D). Like the *mad2*Δ *cdh1*Δ strain, *pds1*Δ *cdh1*Δ cells probably died for a variety of different reasons related to which particular chromosomes were lost.

***cdh1*Δ mutants and DNA replication:** Recently, it was shown that the Cdc28p/Clb inhibitor Sic1p is important not only for regulating mitotic exit but also for keeping Cdc28p/Clb activity low during G_1, which allows prereplication complexes (pre-RCs) to assemble on DNA replication origins (LENGRONNE and SCHWOB 2002). *sic1*Δ mutant cells have a high rate of plasmid and chromosome loss not because of segregation defects but because excessive Cdc28p/Clb activity interferes with origin firing, slowing DNA replication. The delay escapes checkpoint surveillance and cells attempt to separate their sister chromatids while replication intermediates are still present on the DNA. Because Cdh1p cooperates with Sic1p to inhibit Cdc28p/Clb activity during G_1, we considered the possibility that chromosome loss in *cdh1*Δ mutants might also be due to replication defects.

If chromosome loss in *cdh1*Δ and *sic1*Δ mutants has a common cause, then overexpression of *SIC1* might compensate for the lack of *CDH1* and improve chromosome transmission in *cdh1*Δ mutants. Expression of *SIC1* from a high-copy plasmid does in fact reduce the chromosome loss rate of *cdh1*Δ single mutants more than fivefold from 3.6 to 0.7% (Table 2, right column). *SIC1* overexpression can only partially rescue the chromosome loss in *mad2*Δ *cdh1*Δ double mutants, reducing the rate from 8.3 to 4.6%. Intriguingly, these results suggest that there may be two separate defects contributing to chromosome loss in *cdh1*Δ cells. One defect, responsible for the 3.6% loss rate in *cdh1*Δ single mutants, is not recognized by the spindle checkpoint, which is functional in these cells, but is ameliorated by overexpressing *SIC1*. These phenotypes suggest a defect in replication initiation like that seen in *sic1*Δ mutants. Consistent with this possibility, overexpression of *SIC1* eliminates nearly half of the chromosome loss events in *mad2*Δ *cdh1*Δ double mutants. The second defect, which is likely to stem from a spindle or kinetochore malfunction, accounts for the remaining half of the chromosome loss in *mad2*Δ *cdh1*Δ cells. Chromosome loss due to this defect is not affected by *SIC1* overexpression but is suppressed by an intact spindle checkpoint.

We next performed two assays to look for evidence of replication defects in *cdh1*Δ cells. First, we measured the rate of GCR in *cdh1*Δ cells. GCR, which is characterized by large deletions and nonreciprocal translocations, occurs when cells are unable to repair double-strand breaks by homologous recombination. *sic1*Δ mutants have extremely high rates of GCR (575-fold elevated relative to wild type) because they frequently incur double-strand breaks while attempting to segregate chromosomes that are still undergoing replication (LENGRONNE and SCHWOB 2002). To measure GCR, we integrated *URA3* into the left arm of chromosome V near the *CAN1* locus. Because no essential genes are distal to *CAN1* and *URA3*, cells can survive GCR events that result in the deletion of both marker genes. Such cells can be identified because they will be canavanine and 5-FOA resistant. Consistent with previously reported re-

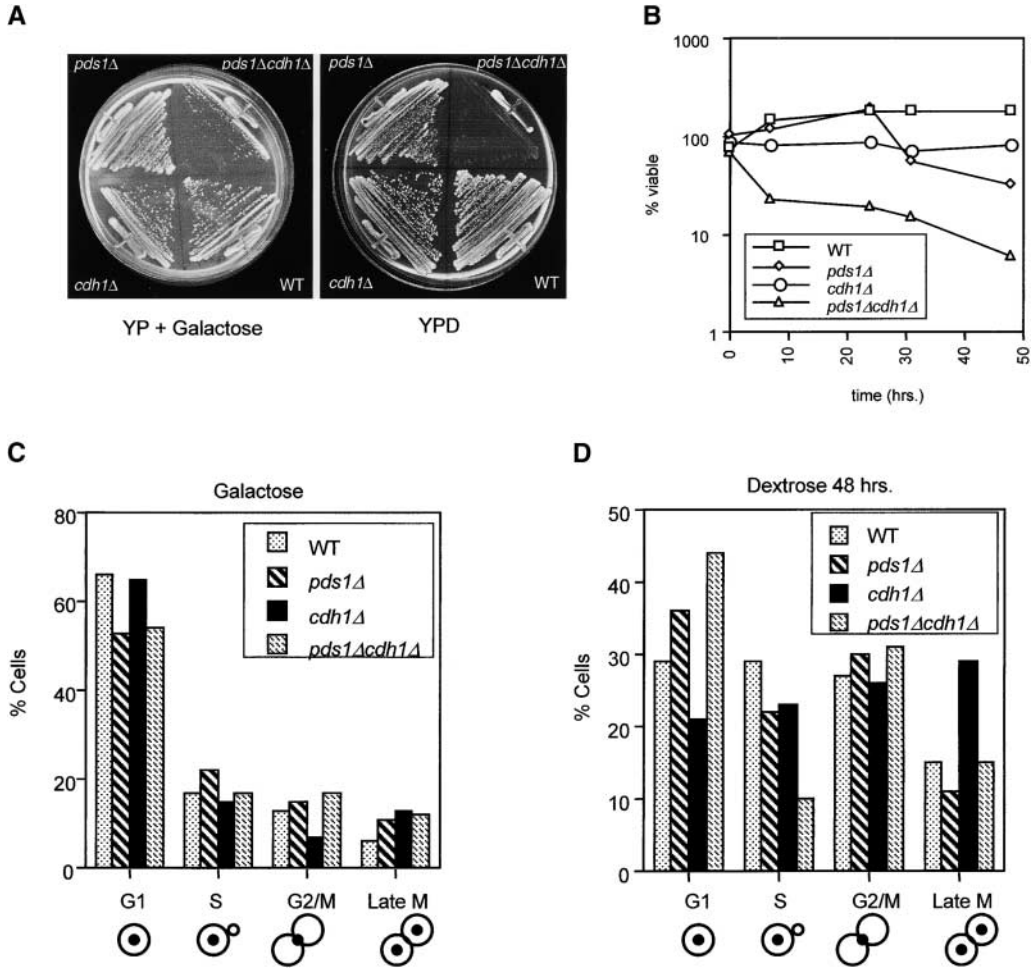

FIGURE 4.—Viability and cell cycle distribution of *pds1*Δ *cdh1*Δ mutants grown in galactose and dextrose. (A) Wild-type (WT; strain KR3056-8A), *pds1*Δ (strain KR3057-18A), *cdh1*Δ (strain KR3054-8D), and *pds1*Δ *cdh1*Δ (strain KR3056-12C) cells were grown at 23° on YP + galactose (YPG, left) or YP + dextrose (YPD; right). (B) Wild-type (WT; strains KR3055-10A and KR3056-8A), *pds1*Δ (strains KR3057-7C and KR3057-18A), *cdh1*Δ (strains KR3054-8D and KR3056-1C), and *pds1*Δ *cdh1*Δ (strains KR3056-1A and KR3056-12C) cells were grown at 23° to midlog phase in YP + galactose liquid medium, transferred to YP + dextrose liquid (0 hr), and grown for an additional 48 hr with dilutions as necessary to maintain the cultures in log phase. At various times, cells were counted and plated on YPG to determine the percentage of viable cells in the culture. (C and D) Samples from the cultures described in B were collected at 0 hr (immediately before the switch from YP + galactose to YP + dextrose (C) and after 48 hr in YP + dextrose (D). Cells were fixed, stained with DAPI, and observed under the microscope. Cell cycle phase was scored as in Figure 2.

sults (LENGRONNE and SCHWOB 2002), we found that GCR was very high in *sic1*Δ mutants (833-fold elevated relative to wild type; Table 5). In contrast, GCR in *cdh1*Δ cells was nearly the same as in wild-type cells (1.6-fold elevated relative to wild type). Although both *cdh1*Δ and *sic1*Δ cells undergo chromosome loss, the results of this experiment suggest that the loss occurs for different reasons in the two mutant strains.

TABLE 4

Missegregation of chromosome V

Strain	% missegregation of chromosome V	
	Galactose	Dextrose (48 hr)
WT	0	0
*pds1*Δ	0	2.1
*cdh1*Δ	1.0	2.1
*pds1*Δ *cdh1*Δ	5.6	21.7

Wild-type (WT, strains KR3055-10A and KR3056-8A), *pds1*Δ (strains KR3057-7C and KR3057-18A), *cdh1*Δ (strains KR3054-8D and KR3056-1C), and *pds1*Δ *cdh1*Δ (strains KR3056-1A and KR3056-12C) cells grown in YP + galactose or in YP + dextrose for 48 hr were scored for missegregation of GFP-tagged chromosome V as described in MATERIALS AND METHODS and text.

Second, we tested whether plasmid loss is suppressed in *cdh1*Δ mutants by increasing the number of origins of replication (ARSs) on the plasmid. Extra ARSs improve plasmid transmission in mutants with defects in replication initiation by increasing the chances that the plasmid will get a competent pre-RC under conditions where pre-RC assembly is difficult (HOGAN and KOSHLAND 1992). We found that a plasmid with a single origin of replication (pDK243) was lost approximately twofold more frequently than a plasmid with eight ARSs (pDK368-7) from WT (303), *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ cells (Table 6; HOGAN and KOSHLAND 1992). Because we observed a twofold difference between the loss rates of pDK243 and pDK368-7 in wild-type as well as mutant cells, it is unlikely that this difference reflects a replication defect in the mutants. As a control, we transformed the same pDK243 and pDK368-7 plasmids into a *cdc6-1* strain in which plasmid loss has previously been shown to be suppressed by extra ARSs (HOGAN and KOSHLAND 1992) and a congenic WT strain (A364a) and measured plasmid loss after incubating the strains

TABLE 5

Gross chromosomal rearrangement

Strain	GCR (FOA^r/CAN^r) rate ($\times 10^{-9}$)
WT	5.4 ± 1.8
*sic1*Δ	4500 ± 500
*cdh1*Δ	8.5 ± 2.7

Between 10^9 and 5×10^9 wild-type (WT, strain KR3118), *sic1*Δ (strain KR3123), and *cdh1*Δ (strain KR3124) cells were plated on canavanine/5-FOA plates to determine the frequency of loss of the *CAN1* and *URA3* genes on the left arm of chromosome V. The average ± standard deviation of two or three independent experiments for each strain is shown.

TABLE 6

Effect of ARS number on plasmid loss rate

	% plasmid loss	
Strain	pDK243	pDK368-7
WT (W303)	3.0 ± 1.2	1.4 ± 0.5
*mad2*Δ	3.1 ± 1.8	1.3 ± 0.7
*cdh1*Δ	5.9 ± 2.3	3.3 ± 2.0
*mad2*Δ *cdh1*Δ	10.4 ± 1.9	6.8 ± 2.1
WT (A364a)	1.7 ± 0.5	1.3 ± 0.7
cdc6-1	15.2 ± 3.3	4.6 ± 1.5

The percentage of loss rate per cell division of plasmids with one ARS (pDK243) or eight ARSs (pDK368-7) was determined for wild-type [WT (W303), strains KR3085-1C and KR3085-6C; and WT (A364a), strain 4521-001], *mad2*Δ (strains KR3085-2A and KR3085-15D), *cdh1*Δ (strains KR3085-6C and KR3085-14A), *mad2*Δ *cdh1*Δ (strains KR3085-18A and KR3085-19D), and *cdc6-1* (strain 4525-061) cells as described in MATERIALS AND METHODS and text. WT (W303) is congenic with the *mad2*Δ, *cdh1*Δ, and *mad2*Δ *cdh1*Δ strains; WT (A364a) is congenic with the *cdc6-1* strain. The WT (A364a) and *cdc6-1* strains were incubated at 36° for 3 hr before plating. All other strains were incubated at 23°. The average ± standard deviation of three to five independent experiments for each strain is shown.

at 36° for 3 hr, the restrictive temperature for the *cdc6-1* mutation. In *cdc6-1* cells, there was a 3.3-fold decrease in the loss rate of pDK368-7 as compared to that of pDK243 while there was virtually no difference between the loss rates of the two plasmids in the wild-type cells (Table 6). We also examined *sic1*Δ mutants because LENGRONNE and SCHWOB (2002) reported that the loss rate of pDK368-7 is sixfold lower than that of pDK243 in this strain (HOGAN and KOSHLAND 1992; LENGRONNE and SCHWOB 2002); however, in our hands, there was no significant difference between the loss rates of pDK243 and pDK368-7 in *sic1*Δ cells (loss of pDK368-7 was only 1.7-fold lower than loss of pDK243). We do not know the reason for this discrepancy with the published results. Nonetheless, since we were able to observe an effect of additional ARSs on plasmid loss in *cdc6-1* cells, and because we did not see any effect in *cdh1*Δ or *cdh1*Δ *mad2*Δ cells, we conclude that these experiments do not support a role for Cdh1p in replication initiation.

Finally, *sic1*Δ and *cdh1*Δ mutants differ in their sensitivities to deletion of *PDS1*. While the *pds1*Δ *sic1*Δ strain grew noticeably more slowly than wild type, it was still much healthier than a *pds1*Δ *cdh1*Δ strain (Figure 5). This is in agreement with LENGRONNE and SCHWOB (2002), who found that mutation of *PDS1* and other spindle and DNA replication checkpoint components had little, if any, effect on *sic1*Δ strains. Taken together, our results suggest that although there is some overlap in the mutant phenotypes of *cdh1*Δ and *sic1*Δ and overexpression of Sic1p can compensate for some of the *cdh1*Δ defects, there are also important differences. Thus, Sic1p and Cdh1p may play distinct roles in ensuring genomic integrity.

DISCUSSION

Our fundamental finding in this study is that *cdh1*Δ mutant cells rely on the spindle assembly checkpoint pathway to prevent rampant chromosome loss. The simplest interpretation of our results is that chromosome segregation is disrupted by accumulation of a protein that normally is degraded by APC/C^{Cdh1p}. APC/C^{Cdh1p} has many substrates, including several that are involved in mitotic progression and spindle function, such as Clb2p (SCHWAB *et al.* 1997), the polo-like kinase Cdc5p (CHARLES *et al.* 1998; SHIRAYAMA *et al.* 1998), the septin-associated kinase Hsl1p (BURTON and SOLOMON 2000), the microtubule motor Cin8p (HILDEBRANDT and HOYT 2001), and the microtubule-binding protein Ase1p (JUANG *et al.* 1997; VISINTIN *et al.* 1997), raising the question of which substrate(s) is critical. It is quite possible that to ensure genomic integrity more than one APC/C^{Cdh1p} substrate must be degraded. JUANG *et al.* (1997) showed that overexpression of nondegradable Ase1p when combined with a *mad1*Δ mutation resulted in a spindle assembly checkpoint-dependent mitotic delay and a high rate of mortality. Likewise, overexpression of nondegradable Cin8p caused a metaphase-like arrest with an abnormal spindle morphology, but dependence on the spindle assembly checkpoint was not determined (HILDEBRANDT and HOYT 2001). Finally, overexpression of Cdc5p resulted in growth arrest that was not restricted to a particular cell cycle stage (CHARLES *et al.* 1998). It is not known whether nondegradable Ase1p, Cin8p, or Cdc5p expressed from their endogenous promoters, equivalent to the situation in *cdh1*Δ mutants, would have the same effect, but it is conceivable that failure to degrade one or more of these proteins contributes to chromosome segregation defects.

It is interesting to note that Cdc20p, the Cdh1p homolog that targets substrates to the APC/C at the metaphase-to-anaphase transition, is itself degraded in an APC/C-dependent manner during G_1 and is therefore

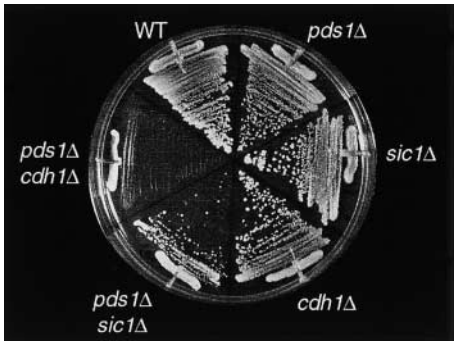

FIGURE 5.—Genetic interaction of *pds1*Δ and *sic1*Δ. Wild-type (strain KR3089-1D, WT), *pds1*Δ (strain KR3089-6C), *sic1*Δ (strain KR3089-1C), *cdh1*Δ (strain KR3055-17B), *pds1*Δ *sic1*Δ (strain KR3089-4D), and *pds1*Δ *cdh1*Δ (strain KR3056-12D; maintained on YP + galactose before streaking onto YP + dextrose) cells were grown on YP + dextrose at 23°.

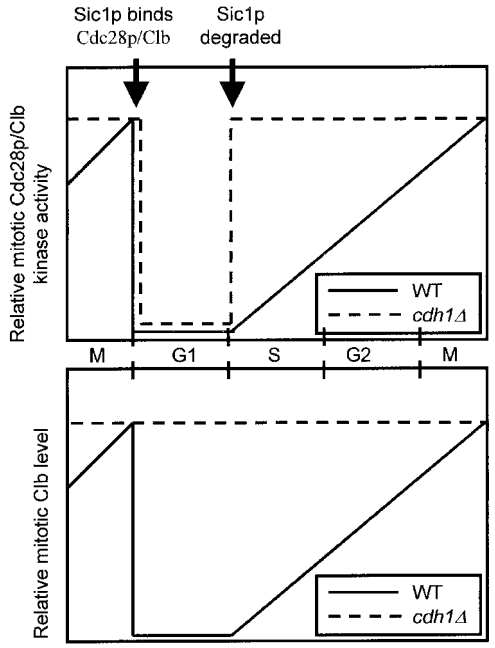

FIGURE 6.—Hypothetical model of the effect of loss of Cdh1p on the profile of Cdc28/Clb activity and Clb levels throughout the cell cycle (see text).

likely to be a Cdh1p substrate (PRINZ *et al.* 1998; SHIRAYAMA *et al.* 1998). Thus, the absence of Cdh1p in *cdh1*Δ mutants may be partially compensated for if Cdc20p is stabilized and APC/C^{Cdc20p} activity persists into late mitosis and G_1.

The fact that the severity of the mutant phenotype in *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ strains is influenced by the level of the Cdc28p/Clb inhibitor Swe1p suggests that the critical proteins requiring degradation in an APC/C^{Cdh1p}-dependent manner are the Clb cyclins. Although it is formally possible that altered Cdc28p/Clb levels do not have a deleterious effect in *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ cells and that Swe1p simply delays the G_2/M transition long enough for the spindle to recover from damage caused by the accumulation of other Cdh1p substrates, we favor the idea that these mutants suffer from abnormal Cdc28p/Clb activity caused by the failure to degrade mitotic cyclins (see below). At the end of mitosis, Clb cyclins are degraded and Sic1p binds to the Cdc28p/Clb complex. These mechanisms appear to have the same end result, inactivation of Cdc28p/Clb, but they are not equivalent. Wild-type cells degrade Clb2p from anaphase onset throughout the next G_1 until APC/C^{Cdh1p} is inhibited by increasing Cdc28p activity at the G_1/S boundary (AMON *et al.* 1994; YEONG *et al.* 2001). From G_1/S through metaphase, *CLB* genes are transcribed and Cdc28p/Clb gradually accumulates (AMON *et al.* 1993). Cells that lack Cdh1p, on the other hand, are likely to maintain high levels of Cdc28p/Clb that is kept inactive by Sic1p during G_1. We hypothesize that when Sic1p is degraded at the onset of S phase, these cells immediately are subjected to high Cdc28p/Clb kinase activity (Figure 6). During much of interphase, *cdh1*Δ mutants may have higher Cdc28p/Clb activity than wild-type cells do, which could lead to inappropriate phosphorylation of certain substrates, acceleration of progression through interphase, and reduced fidelity of some cell cycle events such as kinetochore or spindle assembly. In support of this idea, *cdh1*Δ cells are significantly smaller than wild-type cells, indicating that the balance between cell division and cell growth has been altered (JORGENSEN *et al.* 2002; WASCH and CROSS 2002). One prediction of this model is that artificially reducing Cdc28p/Clb activity, for example, by deleting *CLB2*, should improve the growth of *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ cells. However, because several of the double-mutant combinations adversely affect viability (*e.g.*, *pds1*Δ *clb2*Δ are synthetically lethal) we have not been able to create *pds1*Δ *cdh1*Δ *clb2*Δ and *mad2*Δ *cdh1*Δ *clb2*Δ strains. We have also attempted to compare Clb2p levels and Cdc28p/Clb2p activity in wild-type and *cdh1*Δ cells, but we have not been successful due to difficulties with synchronizing *cdh1*Δ cultures. At this point, we do not know which interphase processes are being derailed in the *cdh1*Δ mutant. Because an effect on cell growth and viability arises only when the spindle assembly checkpoint is compromised, and because *cdh1*Δ mutants that lack the checkpoint have a severe chromosome loss phenotype, we suspect that some aspect of kinetochore or spindle function is impaired. Although we did not notice gross defects in spindle morphology in our *cdh1*Δ mutant, WASCH and CROSS (2002) recently reported that *cdh1*Δ strains do exhibit spindle abnormalities. If the problem lies in kinetochore assembly or in microtubule attachment to the kinetochore, a plasmid with a suboptimal centromere should be especially poorly transmitted in checkpoint-defective *cdh1*Δ cells. In fact, we observed that *mad2*Δ *cdh1*Δ cells lose a plasmid that has a minimal centromere (pRS412; CEN/ADE2; SIKORSKI and HIETER 1989) more than three times more often than they lose

a plasmid that has a centromere that more closely resembles the chromosomal one (pDK243; Table 5; Hogan and Koshland 1992). Wild-type, *mad2*Δ, and *cdh1*Δ cells, on the other hand, lose the two plasmids at approximately the same rate (our unpublished observation). Thus, we speculate that the absence of Cdh1p leads to defects in microtuble-kinetochore attachments in the subsequent cell cycle.

Several explanations may account for why *pds1*Δ *cdh1*Δ mutant strains are less viable and have a more severe chromosome loss phenotype than do *mad2*Δ *cdh1*Δ mutants. First, even though the spindle assembly checkpoint is absent in *mad2*Δ *cdh1*Δ cells, Pds1p still binds and inhibits Esp1p, delaying anaphase for at least the length of time it takes to degrade Pds1p. Cells lacking Pds1p, on the other hand, do not have the protection of this brief delay and may suffer more chromosome loss. Second, Pds1p may indirectly promote the activation of Sic1p: Pds1p enhances the accumulation of Esp1p in the nucleus (Jensen *et al.* 2001; Agarwal and Cohen-Fix 2002); Esp1p, in turn, promotes mitotic exit as part of the Cdc14 early anaphase release (FEAR) network that ultimately leads to the activation of Sic1p and Cdh1p (Visintin *et al.* 1998; Shou *et al.* 1999; Visintin *et al.* 1999). Therefore, *pds1*Δ mutants may have lower FEAR activity than wild-type cells and, consequently, are likely to activate Sic1p less well. Because of the additional effect of reduced Sic1p activity, interphase Cdc28p/Clb activity in *pds1*Δ *cdh1*Δ may be even higher than that in *mad2*Δ *cdh1*Δ, resulting in a more severe chromosome loss phenotype. This study demonstrates that Sic1p and Cdh1p play roles that are overlapping in some respects but distinct in others in maintaining genomic stability. Both *cdh1*Δ and *sic1*Δ mutants lose chromosomes. Chromosome loss in *cdh1*Δ mutants is effectively suppressed by overexpressing *SIC1*, suggesting that a target common to both Sic1p and Cdh1p is likely to be responsible for genomic instability in *cdh1*Δ strains. In *sic1*Δ mutants chromosome loss is due to a replication initiation defect (Lengronne and Schwob 2002). *sic1*Δ cells fail to assemble a full complement of prereplication complexes on origins in G_1, a problem that, intriguingly, escapes the notice of checkpoint systems, and attempt anaphase before replication is complete. This defect accounts for the high rate of GCR in *sic1*Δ mutants and the fact that plasmid transmission in these cells is improved if the plasmid has extra ARSs (Lengronne and Schwob 2002). *cdh1*Δ mutants, on the other hand, are largely indifferent to extra ARSs and have a low rate of GCR, suggesting that they may not have a severe replication defect. Instead, *cdh1*Δ mutants, unlike *sic1*Δ mutants, are sensitive to mutations in the spindle assembly checkpoint, implying that loss of Cdh1p function causes spindle/kinetochore anomalies that can be recognized and corrected in cells with an intact checkpoint.

DNA replication and spindle dynamics are regulated by different Cdc28p/Clb complexes: Clb5p and Clb6p control origin firing and inhibit prereplication complex assembly whereas Clb1p, Clb2p, Clb3p, and Clb4p control later events in G_2 and mitosis. This specialization of Clb function is reflected in the fact that deletion of *CLB5*, but not *CLB2*, partially suppressed many of the defects in *sic1*Δ mutants, including the chromosome loss phenotype (Lengronne and Schwob 2002). The growth of *pds1*Δ *cdh1*Δ and *mad2*Δ *cdh1*Δ strains, on the other hand, did not improve when *CLB5* was deleted nor was plasmid loss in *cdh1*Δ or *mad2*Δ *cdh1*Δ significantly reduced by *CLB5* deletion (our unpublished observations). One caveat to this experiment is that Clb2p, if present at sufficiently high levels, can carry out functions normally driven by Clb5p. Because of the abnormal accumulation of Clb2p in *cdh1*Δ mutants, these cells may continue to have difficulty assembling pre-RCs even in the absence of Clb5p. Even so, this result highlights another way in which *cdh1*Δ and *sic1*Δ cells differ.

There is evidence that regulation of the various subtypes of Clb cyclins differs. Clb5p is primarily a substrate of the APC/C^{Cdc20p}, while Clb2p is predominantly, although not exclusively, ubiquitinated by APC/C^{Cdh1p} (Schwab *et al.* 1997; Visintin *et al.* 1997; Lim *et al.* 1998; Wasch and Cross 2002). Therefore, Cdh1p is likely to have a larger impact on Clb2p levels than on Clb5p levels, which is in keeping with our observation that *cdh1*Δ cells are deficient in Cdc28p/Clb2p-sensitive events like spindle assembly. Conversely, Sic1p has a larger impact on Clb5p as evidenced by the replication defects in *sic1*Δ mutants.

Until now, Cdh1p and Sic1p were thought to be redundant Cdc28p/Clb inhibitors that cooperated to bring about mitotic exit. Our study shows that Cdh1p and Sic1p are each independently important to modulate Cdc28p/Clb activity for successful execution of processes throughout the cell cycle.

We thank Ritu Agarwal, Janet Burton, Joseph Campbell, April Robbins, and Mark Solomon for helpful discussions and critical reading of the manuscript. We also thank Angelika Amon, Sue Biggins, Jake Harrison, Eileen Hogan, Doug Koshland, Kim Nasmyth, and Mark Solomon for strains and plasmids. Finally, we thank Fred Cross for pointing out that *pds1*Δ *cdh1*Δ strains are viable on media containing galactose. This work was supported by a Fellow award from the Leukemia and Lymphoma Society to K.E.R. (no. 5097-02) and by an intramural grant from the National Institutes of Health to O.C.F.

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Communicating editor: B. Andrews