mcl1\(^+[\text{mcl1-1}]^{+}\), the Schizosaccharomyces pombe Homologue of CTF4, Is Important for Chromosome Replication, Cohesion, and Segregation

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Received 5 April 2002/Accepted 26 June 2002

The fission yeast minichromosome loss mutant mcl1-1 was identified in a screen for mutants defective in chromosome segregation. Missegmentation of the chromosomes in mcl1-1 mutant cells results from decreased centromeric cohesion between sister chromatids. mcl1\(^+[\text{mcl1-1}]^{+}\) encodes a \(\beta\)-transducin-like protein with similarity to a family of eukaryotic proteins that includes Ctf4p from Saccharomyces cerevisiae, sepB from Aspergillus nidulans, and AND-1 from humans. The previously identified fungal members of this protein family also have chromosome segregation defects, but they primarily affect DNA metabolism. Chromosomes from mcl1-1 cells were heterogeneous in size or structure on pulsed-field electrophoresis gels and had elongated heterogeneous telomeres. mcl1-1 was lethal in combination with the DNA checkpoint mutations rad3\(\Delta\) and rad26\(\Delta\), demonstrating that loss of Mcl1p function leads to DNA damage. mcl1-1 showed an acute sensitivity to DNA damage that affects S-phase progression. It interacts genetically with replication components and causes an S-phase delay when overexpressed. We propose that Mcl1p, like Ctf4p, has a role in regulating DNA replication complexes.

Genomic instability arises from inaccuracies in DNA replication, repair, or segregation. Genetic screens in yeasts have identified many factors required for stable chromosome transmission by assessing the loss of a nonessential minichromosome. Such mutants are valuable tools in our efforts to understand the mechanisms of genomic stability, often providing insights into the importance of pathways in chromosome maintenance (21, 35, 42, 43, 53, 63, 67, 78). Recent work on the chromosome transmission fidelity (ctf) mutants (ctf4, ctf7, ctf8, and ctf18) in Saccharomyces cerevisiae reveals a direct link between DNA replication and chromosome segregation through the establishment of sister-chromatid cohesion, i.e., the association between newly replicated strands of DNA (29, 47, 59). In Schizosaccharomyces pombe, sister chromatids are held together by a protein complex minimally composed of Psm1p (Smc1 in S. cerevisiae), Psm3p (Smc3), Psc3p (Scc3), and Rad21p (Scc1) (72, 76). Homologues of these four proteins are found from yeast to humans and appear to be conserved in function (44). The cohesin complex forms a stable association between sister chromatids that facilitates the equal distribution of chromosomes to daughter cells during cell division by constraining the replicated strands from scattering, both before and during mitosis. Additionally, cohesins are important for double-strand break repair and general DNA metabolism; thus, cohesin mutants are sensitive to UV light (UV), \(\gamma\)-irradiation, and replication arrest by hydroxyurea (HU) (39, 73, 86). The regulation of cohesin complexes is essential for eukaryotic genomic stability.

Cohesins are broadly distributed on chromosomes in gap 1 (G1) but become localized after DNA replication to the centromeres and intergenic regions along chromosome arms in yeast (5, 7). Cohesins must be present during replication for cohesion to be established, and expression after DNA replication cannot promote cohesion (80). Thus, the redistribution of cohesins in yeast is believed to result from a replication-mediated establishment of cohesion (79). Further, many replication fork components in yeast affect sister-chromatid cohesion, which supports a direct link between the replication fork and the establishment of cohesion. In S. pombe, temperature-sensitive (Ts) mutants of the replication initiator kinase Hsk1p and the DNA polymerase Eso1p (Pol\(\varepsilon\)/Ctf7) all have defects in sister-chromatid cohesion (61, 71). In S. cerevisiae, the replicative repair polymerase Trf4 (Pol\(\varepsilon\)) and alternative replication factor C (RFC) complex (Ctf18, Ctf8, and Ddc1), as well as the Pol\(\varepsilon\)-interacting protein Ctf4, are both important for cohesion between sister chromatids (29, 59, 83). This subset of replication proteins that affects cohesion has led to the proposal that a specific replisome configuration is required to recruit cohesion complexes and establish sister-chromatid cohesion. This model posits that the processive polymerase (Pol\(\varepsilon\)) is replaced with a replicative repair polymerase, such as Pol\(\varepsilon\), for replication of topologically constrained regions of the genome (82). Polymerase switching is proposed to recruit the machinery required for the establishment of cohesion through a yet-to-be-described mechanism. One key part of this switch is the regulation of polymerase access to the primer-template junction; this could very well be a role for the alternative RFC complex (Ctf18, Ctf8, and Ddc1) and Ctf4.

CTF4 was identified in budding yeast by two distinct approaches: a genetic screen for mutants affecting chromosome transmission fidelity (CTF4/CHL15) and affinity chromato-
phy with Polo to identify polymerase one binding proteins (POBI) (30, 41, 48). The stoichiometry of Ctf4-Polo coprecipitation from yeast extracts suggests that these polypeptides associate either weakly or transiently in vivo. Deletion of CTF4 from the budding yeast genome improves the recovery of an essential chromatin-remodeling complex, Cdc68/Pob3p, by Polo affinity chromatography, suggesting that Ctf4 inhibits Cdc68/Pob3p heterodimer binding to Polo. This result has been interpreted to suggest that CTF4 may regulate the assembly of Polo complexes (84). Although CTF4 is not an essential gene for vegetative growth in S. cerevisiae, loss of its function is lethal in combination with many mutations in DNA replication components, such as cf18Δ. This loss also leads to hypersensitivity to methyl methanesulfonate (MMS), suggesting that Ctf4p is important for tolerating replication or genotoxic stress (22). Like most replication mutants, ctf4Δ delays cell cycle progression, while cells have large buds with 2N DNA content, and the nucleus is positioned in the bud neck (36). ctf4Δ viability is also adversely affected by the DNA damage checkpoint kinase mutant mcl1-1 (48). Surprisingly, the ctf4Δ cell cycle delay is alleviated by the deletion of MAD2, a spindle assembly checkpoint component, suggesting that this delay arises in mitosis primarily from a defect in cohesion (29). However, the importance of a mitotic delay for ctf4Δ survival has not been reported.

For this study, we generated S. pombe mutants that showed an increased frequency of minichromosome loss (mcl). These were screened for two additional phenotypes: a mitotic cell cycle delay is alleviated by the deletion of mcl1-1 mutant, that also displayed defective centromeric cohesion. Complementation of the mutant phenotypes identified a single suppressing gene, mcl1Δ, which showed significant sequence similarity to a family of eukaryotic proteins that includes Ctf4p, sepB, and AND-1 from Xenopus sp. and humans. The mcl1-1 mutant has a phenotype mutants, similar to those of sepB3 and ctf4Δ, such as defects in DNA metabolism and cell cycle progression. Our results also suggest the Mcl1p acts in DNA replication as a regulator of postreplication initiation.

MATERIALS AND METHODS

S. pombe strains and methods. The strains used in this study are listed in Table 1. Cells were grown in yeast extract with supplements (YES) or in Edinburgh minimal medium supplemented with the appropriate nutrients (49). Genetic interactions were assessed through standard mating techniques at permissive temperatures, followed by tetrad dissection on YES plates. Transformations were done by lithium acetate or electroporation methods (20, 34). Plasmid-based expression of epitope-tagged proteins was under the control of the mnt1 promoter in the pREP series of vectors; its expression was repressed with 5 μM thiamine (16).

Screening for minichromosome loss mutants. Ten independent SBP32590 cultures were treated with 2% ethyl methanesulfonate until 20% viable, washed extensively in 50% sodium thiosulfate, and then plated onto Edinburgh minimal medium supplemented with uracil, leucine, and low adenine (10 μg/ml). A total of 60,000 cells were screened for red-white colony sectoring, which is indicative of minichromosome loss (26a). A total of 256 sectoring mutants were back-crossed three times to strains SBP32590 and 544 (Table 1), resulting in 40 consistently sectoring mutants.

Light microscopy. Cells fixed with 4% formaldehyde–0.2% glutaraldehyde were characterized by using standard procedures for immunofluorescence (28). Microtubules (MTs) were stained with the 4A1 antibody (54), septal material was stained with Calcofluor (Sigma, St. Louis, Mo.), and DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI).

Flow cytometry. For the flow-cytometric quantification of DNA, 1 ml samples taken from cultures at the time points indicated were washed twice in sterile distilled H2O. Cells were prepared for flow cytometry (fluorescence-activated cell sorter [FACS]) as described in reference 1 by using propidium iodide to stain the DNA nucleic acids.

Treatments with TBZ, HU, UV, γ-ray, and MMS. Cells in liquid cultures containing 12 mM HU or 100 μg of thiabendazole (TBZ)/ml were arrested for <3 h at the specified temperatures unless otherwise indicated. Sensitivities to HU and MMS were assayed by using cells in early log phase (OD450 = 0.3 to 0.6). To assess viability, cells were washed, diluted 1/10,000, and plated to determine the number of viable CFU as a function of increasing exposure. In plating assays, serial dilutions from 10−1 to 10−6 cells/ml for each culture were applied to a set of YES control plates or a set of plates containing YES plus either TBZ (5, 10, 12.5, and 20 μg/ml), HU (1, 2, 3, 4, and 5 mM), or MMS (0.005 and 0.0025%). Plates were incubated for 4 days at 25°C.

For γ-irradiation, 1.5-ml liquid YES cultures were plated in a rotating cylinder and exposed to a 137Cs source emitting 3.7 Gy/min. Samples were taken every hour for 5 h, during which 1,110 Gy was emitted. Viability was determined as described above, by counting the number of viable CFU as a function of increasing exposure of the culture. Viability was measured by plating early-log-phase cells diluted to 1,000 cells/ml in YES onto YES agar plates. Plates were allowed to dry and then exposed to appropriate amounts of UV in a Pharmacia Stratallinker (Peapack, N.J.) equipped with a photodiode detector.

Chromatin binding assays were performed as described by Kearsey et al. (38).
TABLE 1. Yeast strains

| Strain       | Genotype                                      | Source   |
|--------------|-----------------------------------------------|----------|
| SBR32590     | h⁴ ade6.704 leu1-32 ura4-294/pSP (CEN1-7L) ura4⁺ sup3-5 | L. Clarke |
| 544          | h⁴ ade6.704 leu1-32 ura4-294/pSP (CEN1-7L) ura4⁺ sup3-5 | This study |
| 545          | h mcl1-1 ade6.704 leu1-32 ura4-294/pSP (CEN1-7L) ura4⁺ sup3-5 | This study |
| 546          | h mcl1-1 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 99           | h ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 100          | h ade6-M216 leu1-32 ura4-D18 his3-D1 | This study |
| 53           | h cdcl0-129 leu1-32 | | |
| 59           | h weel-50 leu1-32 | | |
| 150          | h cdcl25-22 leu1-32 | | |
| 246          | h cut2-1 ade6-M216 his3-D1 leu1-32 | P. Nurse |
| 494          | h his² lac-FGP lys1¹ lacO leu1-32 ura4-D18 | M. Yanagida |
| 547          | h mad2Δ::ura4⁻ ade6-M216 leu1-32 ura4-D18 | S. Sazar |
| 549          | h bub1Δ::ura4⁻ ade6-M216 leu1-32 ura4-D18 | J. P. Javarzet |
| 1395         | h rad26-T12 ade6-704 leu1-32 ura4-D18 | A. Carr |
| 1123         | h rad26Δ::ura4⁻ ade6-704 leu1-32 ura4-D18 | A. Carr |
| FY369        | h cdcl24-M38 leu1-32 ura4-D18 | S. L. Forsburg |
| FY421        | h chk1Δ::ura4⁻ ade6-704 leu1-32 ura4-D18 | S. L. Forsburg |
| FY865        | h cdsl1Δ::ura4⁻ leu1-32 ura4-D18 | S. L. Forsburg |
| FY945        | h hscl1-1312 ade6-M210 leu1-32 ura4-D18 | S. L. Forsburg |
| FY1105       | h radΔ::ura4⁻ ade6-M216 leu1-32 ura4-D18 | S. L. Forsburg |
| Sp358        | h rpl1Δ::ura4⁻ ade6-M210 leu1-32 ura4-D18 | G. Freyer |
| Sz123        | h rpl1-K547I ade6-M210 leu1-32 ura4-D18 | G. Freyer |
| NA74         | h rad18-74 ade6-704 leu1-32 ura4-D18 | M. J. O’Connell |
| 550          | h /h mcl1Δ::ura4⁻/mcl1⁺ ade6-M210/M216 leu1-32 his3-D1 ura4-D18 | This study |
| 551          | h mcl1-GFP::ura4⁺ ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 552          | h mml1-GFP::ura4⁺ rad1Δ-K1::ura4⁺ ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 553          | h mcl1-GFP::ura4⁺ cds1Δ::ura4⁻ ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 554          | h mcl1-GFP::ura4⁺ mnt1::GSTcds1 LEU2 ade6-704 leu1-32 ura4-D18 | This study |
| 555          | h mcl1-GFP::ura4⁺ hscl1-1312 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 556          | h mcl1-GFP::ura4⁺ cdcl21-M68 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 557          | h mcl1-GFP::ura4⁺ opr1-4 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 558          | h mcl1-GFP::ura4⁺ cdcl22-M45 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 559          | h mcl1-GFP::ura4⁺ cdcl17-K4 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 560          | h mcl1-GFP::ura4⁺ cdcl25-22 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 561          | h mcl1-GFP::ura4⁺ cdcl10-129 ade6-M210 leu1-32 ura4-D18 his3-D1 | This study |
| 562          | h mcl1-1 cds1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18 | This study |
| 563          | h mcl1-1 chk1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18 | This study |
| 564          | h mcl1-1 chk1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18 | This study |
| 565          | h mcl1-1 chk1Δ::ura4⁺ chk1Δ::ura4⁺ ade6-M210 leu1-32 ura4-D18 | This study |
| 570          | h mcl1-1 rad26-T12 ade6-M216 leu1-32 ura4-D18 | This study |
| 571          | h mcl1-1 rad18-74 ade6-704 leu1-32 ura4-D18 | This study |
| 572          | h mcl1-1 rad28Δ::ura4⁻ ade6-704 leu1-32 ura4-D18 | This study |
| 573          | h mcl1-1 radΔ::ura4⁻ ade6-704 leu1-32 ura4-D18 | This study |
| 574          | h mcl1-1 radΔ::ura4⁻ his3-D1 leu1-32 ura4-D18 | This study |
| 575          | h mcl1-1 bub1Δ::ura4⁺ leu1-32 ura4-D18 | This study |
| 576          | h mcl1-1 rad26-T12 ade6-704 leu1-32 ura4-D18 | This study |
| 577          | h mcl1-1 cdcl10-129 leu1-32 | This study |
| 578          | h mcl1-1 weel-50 leu1-32 | This study |
| 579          | h mcl1-1 cut4-533 | This study |
| 580          | h mcl1-1 cut4-665 | This study |
| 581          | h mcl1-1 cut1 | This study |
| 582          | h mcl1-1 cut2 | This study |
| 583          | h mcl1-1 cut2 | This study |
| 584          | h mcl1-1 cdcl25-22 leu1-32 | This study |
| 585          | h mcl1-1 cdcl17-K42 ade6-M210 leu1-32 ura4-D18 | This study |
| 586          | h mcl1-1 pol1-1 ade6-704 leu1-32 ura4-D18 | This study |
| 587          | h mcl1-1 orp1-4 ade6-M210 leu1-32 ura4-D18 | This study |
| 588          | h mcl1-1 cdcl22-M45 ade6-M210 leu1-32 ura4-D18 | This study |
| 594          | h mcl1-1 his7⁺ lac1-GFP lys1⁺ lacO leu1-32 ura4-D18 | This study |

Cdc mutants carrying mcl1-GFP::ura4⁺ were synchronized for chromatin extraction by shifting the cultures to 36°C for 3 h.

Pulsed-field gel electrophoresis (PFGE) was performed as described by Birkenbihl and Subramani (6), except the gel-running conditions were 2 V/cm at an angle of 106°, switching polarity every 1,800 s for 83 h in 0.5X Tris-acetate-EDTA at 16°C, exchanging the buffer every 24 h. After electrophoresis, chromosomes were stained with ethidium bromide and visualized with UV B.

Kinase assays. Protein lysates were prepared by glass beadashing. Immuno-precipitations were carried out with 16B12 monoclonal antibodies (Babco, Berkeley, Calif.), and immunoglobulin G complexes were precipitated with Pan-sorbin (Calbiochem, La Jolla, Calif.). Phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, and 20 μM sodium vanadate) were included in initial washes but were removed prior to kinase assays. Kinase assays were performed as described previously (61), except that GST-Wec1p (1 to 70 amino acids [aa]) was used as the kinase substrate (8).

RESULTS

Screening of mcl mutants identified mcl1-1. mcl1-1 was recovered in the screening of 60,000 ethyl methanesulfonate-mutagenized cells by looking for increased rates of mini-
chromosome loss by a colony-sectoring assay at 25°C. This screening produced 40 consistently sectoring mutants (see Materials and Methods) that we further screened for unequal chromosome segregation by fluorescence microscopy. Fifteen such mutants were tested for genetic interactions with the spindle assembly checkpoint mutant *bub1Δ*. This stringent screening recovered only one mutant, *mcl1*-1, which grew slowly at 25°C and was restricted for growth at 36°C.

Fixed *mcl1*-1 cells examined by fluorescence microscopy showed five categories of aberrant mitotic phenotypes: septation prior to chromatid segregation (Cut) (1), an unequal assortment of DNA to daughter cells (2), lagging chromosomes (3), asymmetric spindle placement within the cell (4), and chromatin bridges (5) (Fig. 1A). The percentage of mitoses with these phenotypes is presented graphically in Fig. 1B. The mitotic index of *mcl1*-1 cells grown at 25°C was not significantly different from a parental control strain’s mitotic index at 25°C (8.6% compared to 8.1%, respectively). However, the mitotic index of *mcl1*-1 cells decreased to 4.3% after 8 h at 36°C while that of control strains remained unchanged. This result suggests that *mcl1*-1 cells have a premitotic arrest of the cell cycle at the restrictive temperature.

These five categories of mitotic phenotypes could represent pleiotropy or gradations of a chromosome loss phenotype. We used live cell observations to distinguish between these possibilities by determining patterns of progression for the static phenotypes observed in the fixed material. Forty living *mcl1*-1 cells were characterized during mitosis by fluorescence microscopy for 30 min or until telophase. Fourteen of the 40 carried out aberrant mitoses that exhibited multiple abnormal phenotypes. These mitoses contained chromatid bridges that commonly progressed to lagging chromosomes. Typically, these cells stopped mitotic progression with mitotic spindles that looked misplaced within the cell (Fig. 1C), and all the cells with lagging chromosomes remained arrested with an elongated spindle for the duration of the observation, suggesting a mitotic checkpoint arrest (25).

The third stage of the screen revealed that *mcl1*-1 shows synthetic interactions with the spindle assembly checkpoint mutant *bub1Δ*. Tetrads dissected from crosses between these two mutants generated microcolonies that were determined to be double mutants (Fig. 1D); only two double-mutant colonies propagated beyond initial colony formation. These strains had a 10-fold decrease in viability compared to that for *mcl1*-1 cells, as determined by the plating efficiency at 25°C (data not shown). To better understand the importance of the spindle assembly checkpoint pathway, we assessed genetic interactions with the known downstream targets of the spindle assembly checkpoint (Table 2) and found interactions only with the cohesin mutant *rad21-K1*, which was synthetically lethal with *mcl1*-1.

*mcl1*-1 cells missegregate endogenous chromosomes due to a defect in sister-chromatid cohesion. The extent and nature of endogenous chromosome loss were measured by studying *mcl1*-1 and *mcl1*-1 strains carrying a tandem array of lac operator (*lacO*) repeats integrated at the *hys* locus and also expressing a lacI-GFP chimera that binds to the *lacO* repeats. This construct acts as a visual marker for the centromere-proximal region of chromosome I (74). A single spot of GFP fluorescence is visible in wild-type cells during interphase; this separates into two during mitosis. We scored chromosome segregation patterns in postmitotic cells prior to cytokinesis so that we could see both daughter nuclei in a single cell. In log-phase cultures at 25°C, 1.5% of the 200 binucleate *mcl1*-1 cells observed showed a 1:0 segregation of the GFP-marked chromosome, suggesting either chromosome loss or nondisjunction. A quantity of 6.2% showed a 2:0 segregation, suggesting sister-chromatid separation without segregation. There were 9.8% that had a Cut phenotype with separated sister chromatids, and 2% had more than two centromeric GFP spots. In an equal number of isogenic *mcl1*-1 binucleate cells, no missegregation event was observed (Fig. 1E and F).

The 2:0 and Cut-chromosome segregation patterns, as well as synthetic lethality with the cohesin mutant *rad21-K1*, suggested that sister chromatids were separating prematurely, leading to failed mitoses. Therefore, we examined preanaphase cohesion in cen1-GFP-marked strains. Cultures at 25°C were treated with 12 mM HU in late S phase and then released into 100 μg of TBZ/ml for 1 h to block mitotic entry. A total of 96.5% of the *mcl1*-1 cells contained a single GFP spot, and 3.5% had two GFP spots separated by ~1 μm by fluorescence microscopy (*n* = 1,588). In contrast, 14.9% of the *mcl1*-1 cells contained two GFP spots per nucleus separated by an average distance of ~2 μm (*n* = 1,122). Representative images are presented in Fig. 1G.

The decreased cohesion seen in *mcl1*-1 cells should promote an increased sensitivity to TBZ, since sister-chromatid separation will be exacerbated by TBZ arrest and should be fatal upon return of the mitotic spindle, as is found in *rad21-K1* cells (73). *mcl1*-1 cells were unable to grow in 12.5 μg of TBZ/ml (Fig. 1H). This sensitivity is similar to that found in the spindle pole body mutant *cut12-1* and greater than that found in the spindle checkpoint mutant *mad2Δ* (11, 32).

**Mcl1p is a member of a conserved protein family.** Only two genomic plasmids were recovered through our complementation analysis; both contained the same 2,548-bp ORF (SPAPB17.02c; accession number AL590605). Northern blot analysis confirmed that a corresponding ~2.8-kbp message was present in equivalent amounts throughout the cell cycle (data not shown). We integrated *arad4* 1.5 kb 3’ of the ORF and demonstrated linkage between this locus and the *mcl1*-1 mutant’s Ts phenotype in both tetrad and random spore analysis. We sequenced the *mcl1*-1 mutant locus and found a single mutation, a guanine-to-adenine transition, 372 nucleotides into the ORF. This changes a TGG (Tyr) to TGA (stop), which prematurely terminates translation after 124 aa.

*mcl1* encodes an 815-aa, highly acidic (pI 4.9), 90.9-kDa protein with similarity to the previously described fungal genes *sepB* from *Aspergillus nidulans* and *CTF4* from *S. cerevisiae* (30, 41, 48). Highly similar proteins were also detected by BLAST searches (3) in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Neurospora crassa*, *Xenopus laevis*, and humans (AND-1), suggesting a conserved eukaryotic family of proteins. Protein domain analysis of Mcl1p by using Pfam identified five β-transducin—also known as WD40 repeat—domains at the N terminus, a motif common to all members of this putative protein family (Fig. 2A) (62). Clustal X alignment of putative family members revealed three highly conserved domains that we refer to here as the *sepB* boxes. These domains appear in a consistent order across the phyla and are
FIG. 1. 
mcl1-1 shows defects in chromosome segregation and cohesion. (A and B) Mitotic phenotypes are identified in log-phase cultures of strains SPB32590 (wild type [wt]) and 545 (mcl1-1) fixed at permissive temperature (25°C) or of strain 545 grown for 8 h at the restrictive temperature (36°C). Panel A is a gallery of representative images for the five categories of mitotic phenotypes presented graphically in panel B. ch, chromatin. (C) mcl1-1 cells expressing α-tubulin–GFP were grown on YES agar pads. The top row of images shows GFP fluorescence, and the bottom row shows chromatin stained with Hoechst 33342. Images represent an elapsed time of 30 min. Bar, ~3 μm. (D) Twelve tetrads from mcl1-1 (strain 546) cross with bub1Δ::ura4+ (strain 549), photographed after 4 days of growth on YES agar at 25°C. Genotypes were determined
TABLE 2. Genetic interactions

| Mutant          | Function                       | Interaction                |
|-----------------|--------------------------------|----------------------------|
| Spindle checkpoint |                               |                            |
| mcl1Δ           |                                |                            |
| bab1Δ           | Checkpoint component           | Loss of viability upon passage |
| cut4            | Checkpoint kinase              | No interaction              |
| cut9            | Anaphase-promoting complex     | No interaction              |
| cut2            | Anaphase-promoting complex     | No interaction              |
| cut1            | Securin                        | No interaction              |
| rad2-1K1        | Cohesin                        | Synthetic lethality         |
| DNA damage checkpoint |                             |                            |
| rad3Δ           | Checkpoint kinase              | Synthetic lethality         |
| rad2ΔA          | Checkpoint component           | No interaction              |
| rad2-6A         | Checkpoint component           | No interaction              |
| rad1Δ           | PCNA-like checkpoint protein   | Decreased permissive temp  |
| rad9Δ           | PCNA-like checkpoint protein   | Decreased permissive temp  |
| rad18-74        | Recombination SMC              | Decreased permissive temp  |
| cds1Δ           | Replication checkpoint kinase  | Partial rescue              |
| chklΔ           | G2 checkpoint kinase           | No interaction              |
| mik1Δ           | cdc2-inhibiting kinase         | Decreased permissive temp  |
| wee1-50         | cdc2-inhibiting kinase         | Decreased permissive temp  |
| cdc25-22        | cdc2-activating phosphatase    | cdc phenotype at permissive temp |
| Replication components |                     |                            |
| pol1-1          | α-Polymerase                   | Decreased permissive temp  |
| orp1-4          | Origin recognition component   | No interaction              |
| cdc17-K42       | DNA ligase                     | No interaction              |
| cdc22-M45       | Ribonucleotide reductase       | No interaction              |
| cdc21-M68       | DNA helicase subunit           | Decreased permissive temp  |
| hsk1-1312       | DNA replication initiation     | Synthetic lethality         |
| rad12-Δ         | RecQ homologue                 | Synthetic lethality         |
| rad12-K1        | RecQ helicase dead mutant      | Synthetic lethality         |
| cdc24-M38       | Essential replication component| Synthetic lethality         |
| dna2-1          | Helicase/exonuclease           | OP partial rescue          |

*a Decreased maximum permissive temperature of mcl1-1 by 3°C.
*b dna2* overproduction (OP) in mcl1-1 cells rescued MMS sensitivity and increased maximum permissive temperature by 3°C.

unique to this family of proteins, distinguishing it from the plethora of other β-transducin or WD40 proteins (Fig. 2B).

**mcl1** is an essential gene. The premature termination of the mcl1-1 allele would be predicted to create a null allele, suggesting that mcl1*, like CTF4, is not essential. We determined the null phenotype by replacing mcl1* with the ura4* gene to generate mcl1Δ:ura4Δ mcl1* diploids (see Materials and Methods). When these strains were sporulated and tetrads were dissected onto complete medium, no more than two of the four spores formed colonies after a week of growth (Fig. 3A, panel 1). Replica plating to medium lacking uracil confirmed that none of the colonies contained the mcl1Δ:ura4Δ null allele (Fig. 3A, panel 2). Examination of the presumptive mcl1Δ spores under a dissection microscope revealed that the null spores had germinated but grown extremely slowly, forming microcolonies of long, cdc-like cells after a week of growth (Fig. 3A, bottom image).

The mcl1Δ phenotype was further characterized by selective germination in medium lacking uracil. Microscopic examination of fixed, DAPI-stained mcl1Δ cells after 48 h revealed that most cells became Cut and had stretched chromatid or lagging chromosomes similar to those found in the mcl1-1 mutant (Fig. 3B). By 72 h, half the cells in the culture had no visible DAPI-stained nuclear material. Flow cytometry showed that mcl1Δ cells germinated and went from 1N to 2N, with kinetics similar to those of the wild-type cells, but they progressively became aneuyploid by 48- and 72-h growth (Fig. 3B, bottom panels). These data suggest that the mcl1-1 allele is not equivalent to a null mutation but must retain some residual function, either in the truncated protein product or as a result of a low level of translational read-through of the premature stop.

Mcl1-GFP is a constitutive nuclear protein that associates with chromatin from G1 to S phase. In S. cerevisiae, Ctf4p interacts with Polα and is present in a constant amount by replica plating and are given as follows: wt, mcl1* bab1*; b, bab1Δ:ura4 mcl1*; m, mcl1-1Δ bab1*; bm, bab1Δ:ura4 mcl1-1*). (E, F, and G) Differential interference contrast (top panels) and deconvolution microscopy with fluorescence optics (lower panels) were used to image living mcl1-1 (strain 594) and mcl1Δ (strain 494) cells with a marked centromere, centromere 1. (E) Binucleate cells show 2:0 and 1:1 segregation. (F) Segregation patterns were categorized as follows: one GFP spot in only one nucleus of two (1:0), one GFP spot in both nuclei (1:1), two GFP spots in only one nucleus of two (2:0), no segregation (cut), or more than two GFP spots present (other). (G) Representative images from the sister-chromatid cohesion study are shown. (H) Serial dilutions of mcl1-1 (strain 546), mcl1* (strain 100), mcl1-1Δ (strain 547), and cut12-1 (strain 246) plated onto YES agar and YES agar plus 12.5 μg of TBZ/ml. Bars, 5 μm.
throughout the cell cycle and it associates with chromatin from the S to M phases of the cell cycle (29). To determine if Mcl1p localizes similarly, we used a mcl1-GFP chimera expressed from the endogenous mcl1/H11001 promoter. Mcl1-GFP strains were wild type for all mcl1-1 phenotypes and showed constitutive nuclear localization. GFP fluorescence appeared excluded from at least part of the nucleolus, based on the absence of fluorescence from the region occupied by the ribosomal DNA (rDNA) bars that protrude from the bulk of the chromatin in fission yeast (Fig. 4 [the red crescent is Hoechst staining of recently deposited cell wall material]). Live cells expressing the chimera showed no variation in protein localization throughout the cell cycle, but a large fraction of Mcl1-GFP appeared to be freely diffusible within the nucleus as it filled any deformation of the nuclear envelope (Fig. 4B).

Soluble Mcl1-GFP was extracted from the nuclei of detergent-permeabilized cells to visualize any structure-bound material (see Materials and Methods). In log-phase cells, Mcl1-GFP colocalized with DAPI-stained DNA and retention of the GFP signal correlated with the stage in the cell cycle. Mcl1-GFP appeared structure bound and colocalized with chromatin predominantly in binucleate G1/S cells, but it was washed from chromatin in cells with a single nucleus (G2) and was absent from all nuclei that contained a mitotic spindle (Fig. 4C).

Nuclear retention of Mcl1-GFP was abolished by DNase I digestion of permeabilized cells, followed by the same extraction (Fig. 4C).

Since this result differs from the reported localization of Ctf4p, which is chromatin bound from S to M phase, we examined the relationship between cell cycle and Mcl1-GFP–DNA colocalization more closely. We constructed a series of mcl1-GFP cdc double-mutant strains (cdc10-129 [G1], orp1-4 [pre-replication initiation], cdc22-M45 [early S phase], cdc21-M68 [mid-S phase], cdc17-K42 [late S phase], and cdc25-22 [G2]) and used HU (S phase) or TBZ (pre-M phase) to enrich for cells at specific cell cycle stages prior to chromatin extrac-

**FIG. 2.** mcl1+ is a member of a eukaryotic protein family. (A) The domain structure of Mcl1p and sequence comparison results among a select set of sepB family members are shown. Identity and similarity percentages are for comparisons over the entire length of the protein by using the Smith-Waterman algorithm (60). HMG, high-mobility group. (B) Clustal W alignment of the CTF4- and sepB-like proteins reveals three domains of high similarity in these aligned peptide sequences (2). S.p., S. pombe; A.n., A. nidulans; S.c., S. cerevisiae; H.s., Homo sapiens.
that mcl1 partial escape from the TBZ arrest during preparation.

25% of the cells in Mcl1-GFP cultures treated for 3 h with 100 g of TBZ/ml exhibited GFP-DAPI colocalization, but of this fraction. These arrested cells showed a gradual loss of Mcl1-GFP from chromatin as the G1/S phase (100% in cdc10-129 mutant) gave way to G2 (18% in cdc25-22 mutant) (Fig. 3D). Indeed, 25% of the cells in Mcl1-GFP cultures treated for 3 h with 100 µg of TBZ/ml exhibited GFP-DAPI colocalization, but of this 25%, a significant number had two nuclei (89%), indicating a partial escape from the TBZ arrest during preparation.

Genetic interactions and synthetic dosage effects indicate that mcl1 affects DNA replication. CTF4 mutants interact with a number of replication components, so we also looked for genetic interactions between mcl1 and various replication mutants (Table 2). Similar to ctf4Δ, we found only moderate interactions between the pol1 mutant and mcl1. The severest phenotypes were found between mcl1 and the DNA replication initiator kinase mutant hsk1-1312, two alleles of the RecQ helicase (rgh1Δ, rgh1-K547I), and an allele of the essential DNA replication factor cdc24-M38, which interacts with PCNA and RFC (17, 26, 61). Additionally, we found that multicopy genomic plasmid containing dna2Δ gave partial rescue of mcl1's phenotypes (26, 69). We found no interactions with cdc21-M68, orp1-1, or cdc17-K42.

mcl1 expression from either pREP3X or multicopy genomic plasmids caused wild-type cells to grow more slowly than under plasmid-repressed or plasmid control conditions. Overexpression of mcl1 led to cdc-like cells that were biased toward <2N DNA content (Fig. 5A and B). S. pombe cells rarely show a 1N DNA content by FACS because cytokinesis is not completed until mid-S phase, making cells appear to have a 2N DNA content throughout the cell cycle. If overexpression of mcl1 indeed causes a DNA replication delay relative to septation, we reasoned that replication mutants that interact with or regulate Mcl1p function or DNA replication might be acutely sensitive to Mcl1p overexpression. Thus, we looked for synthetic dosage phenotypes in replication mutants when Mcl1-PKp was expressed from a medium-strength promoter (pREP41x), a level of expression that causes no detectable phenotypes in wild-type cells. Of the mutants listed in Table 2, only the hsk1-1312 mutant showed synthetic dosage interactions. Viability of hsk1-1312 was not affected by Mcl1-PKp overproduction at 29°C, since both of these strains had a similar plating efficiency (Fig. 4C). However, the hsk1-1312 strain overproducing Mcl1-PKp grew slower and formed only microcolonies in the time sufficient for hsk1-1312 strains carrying empty vectors to form normal-sized colonies. Flow cytometry performed on these two strains grown at 29°C in liquid culture indicated that they contained a similar DNA content until 12 h of induction, when maximal production of Mcl1-PKp was reached (23). By 24 h, excess Mcl1-PKp caused a <2N DNA bias in the population of hsk1-1312 cells whereas the empty vector controls appeared normal with a 2N DNA content (Fig. 5D). Thus, it appears that overproduction of Mcl1p exacerbates the S-phase delay phenotype of hsk1-1312 cells at 29°C (61, 68).

mcl1 affects chromosome integrity. Chromatin bridges were common to mcl1 mitoses, suggesting incomplete DNA replication or poor resolution of sister chromatids. If chromosomes were incompletely replicated in mcl1, then their migration in PFGE gels should be severely retarded, as when wild-type cells are HU arrested (Fig. 6A, lane 2). However, the chromosome-banding pattern for the mcl1-1 chromosomes at 25°C appears not reduced but more diffuse than that of the mcl1 control. This indicates that mcl1-1 chromosomes were...
Mcl1-GFP is a constitutive nuclear protein that associates with chromatin during G1 and S phase but not during mitosis. (A) Hoechst 33342-stained DNA forms a three-quarters circle with two protruding bars, which are the rDNA genes extending into the nucleolus. Mcl1-GFP expressed from its endogenous promoter two protruding bars, which are the rDNA genes extending into the nucleolus. Mcl1-GFP is freely diffusible in the nucleus. (C) Log-phase cells show a redistribution of Mcl1-GFP (top panels) and GFP fluorescence (bottom panels). There was no apparent redistribution of Mcl1-GFP fluorescence through the cell cycle, and any deformation in the nuclear membrane was filled with GFP fluorescence (white arrowheads), indicating that much of the Mcl1-GFP is freely diffusible in the nucleus. (C) Log-phase cells showing MTs in blue, DNA in red, and Mcl1-GFP in green. Using cell morphology to assess the cell cycle stage, we quantified the fraction of cells in which GFP and DNA colocalized after the extraction of soluble protein (see Materials and Methods). The data are presented just below the panel of images (n = 251). The far right panel is an image of cells treated with DNase I and then similarly extracted. The bar represents 5 μm. (D) Chemical and mutant cell cycle arrests were used to confirm log-phase results for the stage-specific localization of Mcl1-GFP to chromatin. We tested the retention of Mcl1-GFP in cdc10-129 (strain 561), orp1-4 (strain 557), cdc22-M45 (strain 558), HU (strain 551), cdc21-M68 (strain 556), cdc17-K42 (strain 559), cdc25-22 (strain 560), and TBZ mutants (strain 551) under arrested conditions (3 h at 36°C for mutants and 3 h at 32°C for chemical arrests). The percentages of cells (n > 200 each) in which GFP signal colocalized with DAPI are presented graphically.
single mutants, suggesting that unrestricted passage of mcl1-1 into or through mitosis was equally lethal.

**mcl1-1 is partially rescued by cds1Δ but is synthetically lethal with rad3Δ and rad26Δ.** We determined the DNA damage checkpoint components important for mcl1-1 viability. In this study, 44 four-spore tetrads were examined for each cross. We were unable to recover the mcl1-1 rad3Δ or mcl1-1 rad26Δ double mutants but did recover the mcl1-1 rad1Δ and mcl1-1 radΔ double mutants, albeit they were very sickly (Table 2). We readily recovered the mcl1-1 cdsΔ and mcl1-1 chkΔ double mutants. The mcl1-1 chkΔ double mutants looked indistinguishable from the mcl1-1 single mutants; however, growth of the mcl1-1 cdsΔ double mutants was more robust. These mutants displayed a shorter G2 than mcl1-1 mutants but were still restricted for growth at 36°C (Fig. 7B). Triplicate mutants were recovered as nonparental ditype tetrads from crosses between the mcl1-1 cdsΔ and mcl1-1 chkΔ strains (backcrosses confirmed the presence of both deletions). Even though spore viability from these meioses was severely decreased, recovered strains looked and grew indistinguishable from the mcl1-1 cdsΔ strains (Fig. 7B). Alleviation of the mcl1-1 mitotic delay phenotype by cdsΔ suggests that the cell cycle delay seen in mcl1-1 cells was principally due to Cds1p but that the delay was not essential for mcl1-1 viability at permissive temperatures.

Cds1p was initially identified in fission yeast as a high-copy-number suppressor of the polo mutant swi7-H4, where it acts to enforce the S/M checkpoint; it is also an S-phase regulator important for replication damage recovery (45, 50). To determine if Cds1p activation in mcl1-1 cells could explain the slow-growth phenotypes observed at permissive temperatures, we constructed mcl1-1 rad26-T12 double mutants. rad26-T12 is defective in Cds1p activation (45), and we expected that mcl1-1 rad26-T12 mutants would be rescued for slow growth if Cds1p were constitutively activated by the mcl1-1 mutation. No synthetic growth phenotypes were seen, suggesting that Cds1p is not activated in mcl1-1 cells, at least not by Rad3p/Rad26p (data not shown). To confirm that Cds1p was not being activated by another mechanism, we assayed Cds1p kinase activity by immunoprecipitation of hemagglutinin (HA) epitope-tagged alleles of cds1+ and cds1-KD (kinase dead) from mcl1-1 and mcl1+ cells (61). Kinase activity was measured with the N-terminal 70 aa of Wee1 fused to glutathione S-transferase (GST) as substrate (8). In both strains, Cds1p kinase activity was undetectable in log-phase cells and in cells carrying the kinase-dead allele but it was highly active following a 3-h incubation in 12 mM HU (Fig. 7C, top panel). This suggested either that Cds1p activation was not responsible for the mcl1-1 slow-growth phenotype or that this assay was too insensitive to

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**FIG. 5.** Overexpression of Mcl1p causes a G1/S arrest. (A) Wild-type cells (strain 100) transformed with pREP3x mcl1-GFP grow slowly when expression is induced. (B) FACS analysis of cells induced for 12 h show a modest G1 accumulation in cells expressing high levels of mcl1-GFP compared with cells repressed for this expression. (C) Expression of Mcl1-PK in the hsk1-1312 (FY594) mutant under inducing conditions severely retarded growth of the mutant in comparison to hsk1-1312 mutant carrying an empty vector. However, this expression does not lead to increased cell death. Cells were grown for 4 days at 29°C; (D) hsk1-1312 cells under inducing conditions for 24 h have a shift to 1N DNA content when expressing mcl1-PK but not in the empty vector controls (C). Expression from the nmt promoter starts at time zero and reaches a maximum after 12 h (23). Data are representative of three independent transformations of hsk1-1312 mutants with pREP41x mcl1-PK1 and pREP41x mcl1-GFP.
detect the low levels of Cds1 activity that may be sufficient for checkpoint delay. Interestingly, we noticed that the induction of either Cds1-HAp or Cds1KD-HAp expression dramatically inhibited mcl1-1 growth (Fig. 7D). This was most apparent in mcl1-1 cells carrying pREP42X cds1-KD HA when comparing induced and uninduced growth at the semipermissive temperature of 32°C. We inferred from this result that Cds1p is a molecular poison to mcl1-1 cells.

DNA metabolism is compromised in mcl1-1 cells. The genetic interactions of mcl1-1 with a specific subset of the DNA damage checkpoint pathway suggested that the product of this locus could be affecting specific DNA repair functions that are essential for normal vegetative growth. To identify affected pathways, we tested mcl1-1 cells for sensitivity to the damage caused by UV, HU, γ-irradiation, and MMS. The mcl1-1 mutant showed only slight sensitivity to UV compared to that for the mcl1+ and checkpoint mutants, suggesting that nucleotide excision repair in mcl1-1 was mostly intact. However, γ-irradiation, which induces double-strand breaks, led to the significantly decreased viability of the mcl1-1 mutant compared to that of wild-type strains. Surprisingly, the mcl1-1 cds1Δ double mutant showed synergistic sensitivity to γ-irradiation compared with that of either single mutant (Fig. 8A). mcl1-1 cells were also hypersensitive to growth in the continual presence of HU. Prolonged HU treatment is believed to cause fork collapse, and mutants sensitive to HU are either defective in S/M checkpoint delay, like rad3Δ, or recovery from this arrest, like rgt1Δ and cds1Δ (56). The mcl1-1 mutant was unable to grow in the continual presence of 3 mM HU. The mcl1-1 cds1Δ double-mutant strains, like rad3Δ, could not grow in the presence of 1 mM HU (Fig. 8B), suggesting synergy between these two mutations in the cellular response to HU. The mcl1-1 and cds1Δ single mutants as well as the mcl1-1 cds1Δ double mutants blocked cell cycle progression as assessed by a decrease in septation index (Fig. 8C and data not shown), but unlike the mcl1-1 mutant alone, which maintained a high viability of up to 6 h (Fig. 8D), the cds1Δ mcl1-1 double mutants showed a similar sensitivity to HU as did rad3Δ and they showed an abbreviated arrest. This implies that Cds1p and Mcl1p function in separate pathways that ensure proper replication arrest and recovery.

Like ctf4Δ, mcl1-1 was also hypersensitive to the DNA alkylating agent MMS, which can block S-phase progression. Overcoming MMS toxicity requires the recruitment of replicative repair polymerases to perform base excision repair (BER) or replication bypass (33, 75). mcl1-1 cells were unable to grow in the continual presence of 0.0025% MMS. This sensitivity was indistinguishable from that of rad3Δ cells (Fig. 8E). We tested log-phase cultures treated with 0.02% MMS for S-phase checkpoint delay effects. Log-phase mcl1+ cells so treated for 4 h showed <60% of the cells delayed in S phase (Fig. 8F, left histograms), whereas treated rad3Δ cells did not delay replication (middle histograms). The mcl1-1 mutant appeared less delayed in S phase than mcl1+ cells (right histograms) and lost viability by 3 h, suggesting that mcl1-1 is defective in BER and perhaps in S-phase arrest as well.

**DISCUSSION**

Mcl1p belongs to a conserved eukaryotic family of proteins found in plants, fungi, and animals that is essential for chromosome maintenance in fungi. This family of proteins contains two recognizable motifs: an N-terminal WD40 domain, followed by three unique, highly conserved sepB boxes. In vertebrate members, there is also a C-terminal high-mobility-group AT hook DNA binding domain (13, 40). Since Ctf4p has only a single WD40 repeat—inaequate to form the β-propeller structure of a G-beta-like molecule—the highly conserved...
sepB boxes represent the only unique feature that is common to all family members and distinguishes this family from the myriad of other WD40 domain proteins. WD40 proteins are typically regulatory and have never been described to possess enzymatic function. Therefore, the sepB domains most likely represent an additional interaction domain unique to this family and possibly to its function.

sepB3, ctf4Δ, and mcl1-1 cells all have strong chromosome missegregation phenotypes, with ctf4Δ and mcl1-1 cells showing defects in sister-chromatid cohesion (Fig. 1). The segregation of chromosomes during mitosis is dependent on sister kinetochores achieving a bipolar attachment to spindle MTs. Kinetochores become so oriented because newly replicated sister chromatids maintain cohesion until tension is generated at kinetochores and anaphase onset is triggered. Tension generated by cohesion is monitored by the spindle assembly checkpoint, and the absence of cohesion has been shown to activate the spindle assembly checkpoint (64). This blocks the cascade of events that initiate the dissolution of cohesion, anaphase onset, and mitotic progression (10). ctf18Δ and ctf4Δ cells exhibit a cell cycle delay dependent on mad2Δ, but it is unknown if this contributes to cell viability (29). In this study, the viability of mcl1-1 cells was dependent on the spindle assembly checkpoint protein Bub1p but not Mad2p. When Bub1p is present, mcl1-1 cells show a mitotic delay in cells that missegregate chromosomes, suggesting that it is the absence of cohesion that triggers the spindle checkpoint and that this is important for mcl1-1 viability. This is further supported by the observation that mcl1-1 was sensitive to the MT poison TBZ and was lethal in combination with the cohesin mutant rad21-K1 (Fig. 1). These multiple observations support the conclusion that mcl1-1 missegregates chromosomes due to defective sister-chromatid cohesion.

Neither sepB3 cells nor mcl1-1 cells accumulate in mitosis, suggesting that cells predominately arrest in G2, which differs from the recent observation that the ctf4-s65 arrest is enforced by Mad2p and is therefore mitotic. S. pombe is an excellent system in which to test the relative importance of the DNA

![Graph](image_url)
damage versus the spindle assembly checkpoints’ impact on cell viability, because of the well-defined G₂-to-M transition and the clear role of available cell cycle mutants. We found passage into or through mitosis to be equally detrimental to mcl1-1 cells at 36°C, suggesting that any progression toward cell division was lethal to mcl1-1 cells. Further, the loss of Mcl1p function must damage DNA in a way that specifically requires the Rad3/Rad26 DNA checkpoint complex for repair and viability, since we found that none of the other DNA damage checkpoint components were essential for mcl1-1 viability. Similar but less severe dependency on the ataxia-telangiectasia mutated and ataxia-telangiectasia-related checkpoint kinases has been described in sepB3 and ctf4Δ cells, where sepB3 delay of septation during conidiation is alleviated by the uvsB110 mutation and ctf4Δ has a dramatically reduced viability in combination with mcl1-1 (30, 31, 48). Chromosomes appear heterogeneous in mcl1-1 cells, and this phenotype is enhanced by growth at 36°C. This is likely to occur both from low replication fidelity, as suggested by the longer, heterogeneous telomeres and from chromosomal shearing during anaphase, seen in chromatin bridges during live cell observations.

The growing number of yeast replication components important for cohesion supports the polymerase-switching model for the replication-mediated establishment of cohesion (29, 47, 59, 68, 70, 71, 77, 83). Trf4p in S. cerevisiae belongs to the β-polymerase family of DNA replication repair polymerases and is required for the establishment of cohesion, as well as for the replication of topologically constrained DNA (82). It has been proposed that the alternative RFC (Ctf8p, Ctf18p, and Ddc1p) complex important for cohesion promotes the loading of Trf4p (Polo) in regions of the genome where replication forks become blocked by chromatin structures, such as sites occupied by cohesins. The relationship between TRF4, this novel RFC complex (which includes Ctf18p), and cohesins has not yet been established, but Ctf4p would be an attractive link between the alternative RFC complex and the primer template since ctf4Δ is synthetically lethal with ctf18Δ and Ctf4p physically interacts with Polo, the replication primase in eukaryotes.

β-Polymerases are essential for the replicative repair of DNA damage, suggesting that mutants defective in β-polymerase switching may also be sensitive to genotoxic stress that requires BER (55). Both ctf4Δ and mcl1-1 cells are hypersensitive to MMS, which is repaired by BER pathways (Fig. 8E) (48). mcl1-1 cells, in comparison to the wild type, do not have a strong S-phase delay in response to 0.02% MMS treatment (Fig. 8F). This hypersensitivity to MMS and a diminished S-phase delay suggest that mcl1-1 cells do not properly respond to MMS damage during replication. Given that Dna2p, which has been reported to be involved in DNA damage repair and lagging strand DNA synthesis (37), can partially rescue this sensitivity when overproduced, mcl1-1 cells may simply be deficient in efficiently recruiting the replicative repair machinery
mcl1-1 has strong interactions with other DNA replication components. mcl1-1 was lethal in combination with cdc24-M38 (Table 2). Cdc24p is involved in polymerase loading, as it genetically and physically interacts with Pcn1p (PCNA) and Rfc1p in fission yeast (69). Moreover, rqh1Δ and rqh1-HD were lethal in combination with the mcl1-1 mutant. Rqh1p, the fission yeast RecQ helicase homologue, is a DNA structure-specific helicase that is important for the replication of topologically constrained regions of the genome and genomic stability (17). These data suggest that mcl1-1 is important for efficient DNA synthesis and possibly for the replication of topologically constrained DNA. Moreover, mcl1-1 in combination with the replication initiator kinase mutant hsk1-1312 allele is lethal. These two mutants independently share a number of phenotypes: both have reduced sister-chromatid cohesion, both are partially rescued by the deletion of cds1+, both are synthetically lethal with rqh1Δ, and both show extreme sensitivity to HU and MMS (61, 68). Unfortunately, the functional relationship between Mcl1p and Hsk1p remains to be determined in this study. The interactions between Hsk1p and Cds1p kinases appear to be complex, as Hsk1p is both a substrate and an activator of Cds1p, though the effect of Hsk1 phosphorylation by Cds1p has not yet been determined (61, 68). Thus, the relationship of Mcl1p to these two known S-phase regulators remains difficult to specify but they nevertheless appear intertwined.

The synergistic sensitivity of mcl1Δ mcl1-1 cells toward HU is similar in degree to that of rad3Δ cells (Fig. 8C), which completely lack a checkpoint response. This result suggests that Cds1p acts in concert with Mcl1p for proper HU tolerance. We also found that the deletion of cds1+ partially rescued mcl1-1 slow growth, suggesting that it is primarily responsible for the late-S or G2 phase delay in mcl1-1 cells. This interpretation requires Cds1 activation in mcl1-1 cultures, but we found no detectable Cds1p activation nor any defect in its activation. We cannot, however, rule out the possibility that our assay was not sensitive enough to detect a Cds1 kinase activity that could be sufficient for checkpoint delay. Given that both kinase-active and kinase-dead alleles of Cds1p can act as molecular poisons to mcl1-1 growth under permissive conditions, we believe Cds1p regulates a pathway that may be deleterious to mcl1-1 viability. One known target of Cds1p, possibly independent of its kinase activity, is the regulation of the Mus81 Holliday junction resolvase (14). If HU-stalled replication forks collapse, the reinitiation of replication from these collapsed forks would require both helicase and resolvase activity, such as that described for Rqh1p and Mus81p (9, 27, 65). Unfortunately, the functional implications of the Cds1p-Mus81p interaction are not known, but these data suggest that S. pombe cells with diminished Mcl1p carry out DNA replication that is sensitive to both the presence of Cds1p and the absence of Hsk1p and Rqh1p.

The best-understood role of the Dfp1p/Hsk1 kinase (DDK) is to initiate DNA replication (12, 46). Therefore, the increased accumulation of cells with less than 2N DNA in an hsk1-1312 culture, when Mcl1-PK is in excess, suggests that Mcl1p can antagonize either processive DNA replication or its initiation. Such a role is supported by our observations that overexpression of Mcl1p in wild-type cells also leads to a cell cycle delay with a less than 2N DNA content. However, this effect at extremely high levels of Mcl1p overexpression in wild-type cultures is not as dramatic as that found in hsk1-1312 cells overexpressing Mcl1p to much lesser extent, possibly because hsk1-1312 cells are already compromised in S-phase progression at 29°C. The Dfp1p/Hsk1p role in DNA replication initiation is believed to result from its phosphorylation of a number of replication fork components, such as Mcm2p (12, 68). We have found no evidence for Mcl1p phosphorylation (our unpublished observations), although some WD40 domain proteins specifically recognize phosphorylated binding partners (85). This leaves open the possibility that Mcl1p may be regulated not by being a substrate for phosphorylation but by recognizing the modification of its binding partner(s).

The identification of Ctf4p as a Polo binding protein suggests a possible mechanism for the blocking of DNA replication. Competition for binding to Polo between Ctf4p and the Cdc68p/Pob3p chromatin-remodeling complex could be a point of regulation for replication fork initiation or progression. Cdc68p/Pob3p and a homologous complex in Xenopus sp. (DNA unwinding factors p87/p140) are essential for efficient replication initiation (52, 58). If Mcl1p, like CTF4, acts to restrict access to Polo, then an excess of Mcl1p in a kinase-compromised Hsk1 mutant would be expected to block postinitiation DNA replication. This hypothesis remains to be tested, however, and will require detailed protein interaction analysis, a current focus in our lab.

Eukaryotic DNA replication is distinct from bacterial DNA replication in that it requires multiple polymerases. Indeed, both DNA repair and recombination require selective recruitment of or access to the templates of specific polymerases (66). The mechanism of genomic instability exhibited by the CTF4, mcl1+, and sepB mutants, as well as the genetic and physical interactions with DNA replication and repair components, suggests that this family of eukaryotic proteins has an important role in regulating polymerase-containing complexes. The importance of these proteins in fungi suggests that further investigation into their response to DNA damage and protein interaction partners may be important to our understanding of polymerase regulation and genomic stability in all eukaryotes.

ACKNOWLEDGMENTS

We thank G. Freyer, S. Sazar, L. Clarke, A. M. Carr, M. J. O’Connell, J. P. Javerzat, I. Hagan, A. Yamamoto, P. Russell, and D. Q. Ding for yeast strains and reagents. We particularly thank Susan Forsburg and her lab for helpful advice, keen interest, and yeast strains.

D.R.W. was supported by training grant GM07135 to W. B. Wood. This work was supported by National Institutes of Health grant GM-33787 to J.R.M., who is a research professor for the American Cancer Society.

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