MCB
Journals.ASM.org

G_1/S and G_2/M Cyclin-Dependent Kinase Activities Commit Cells to Death in the Absence of the S-Phase Checkpoint

Nicola Manfrini, Elisa Gobbini, Veronica Baldo,* Camilla Trovesi, Giovanna Lucchini, and Maria Pia Longhese

Dipartimento di Biotecnologie e Bioscienze, Università di Milano–Bicocca, Milan, Italy

The Mec1 and Rad53 protein kinases are essential for budding yeast cell viability and are also required to activate the S-phase checkpoint, which supports DNA replication under stress conditions. Whether these two functions are related to each other remains to be determined, and the nature of the replication stress-dependent lethality of *mec1* and *rad53* mutants is still unclear. We show here that a decrease in cyclin-dependent kinase 1 (Cdk1) activity alleviates the lethal effects of *mec1* and *rad53* mutations both in the absence and in the presence of replication stress, indicating that the execution of a certain Cdk1-mediated event(s) is detrimental in the absence of Mec1 and Rad53. This lethality involves Cdk1 functions in both G_1 and mitosis. In fact, delaying either the G_1/S transition or spindle elongation in *mec1* and *rad53* mutants allows their survival both after exposure to hydroxyurea and under unperturbed conditions. Altogether, our studies indicate that inappropriate entry into S phase and segregation of incompletely replicated chromosomes contribute to cell death when the S-phase checkpoint is not functional. Moreover, these findings suggest that the essential function of Mec1 and Rad53 is not necessarily separated from the function of these kinases in supporting DNA synthesis under stress conditions.

The integrity of the genome is constantly challenged by DNA damage caused by environmental and intracellular factors. Aberrant DNA replication is a major source of mutations and chromosome rearrangements that can lead to cancer and other diseases in metazoans (reviewed in reference 23). Replication fork progression can be hampered by exogenous or endogenous DNA damage. Furthermore, faithful replication depends on a balanced supply of deoxyribonucleotides (deoxyribonucleoside triphosphates [dNTPs]), whose levels are maintained during S-phase through the action of the ribonucleotide reductase (RNR) activity that converts the ribonucleotides to dNTPs (reviewed in reference 37). Indeed, replication fork pausing can be experimentally induced by genotoxic drugs, such as hydroxyurea (HU), which reduces dNTP pools by inhibiting RNR activity, and the DNA alkylating agent methyl methanesulfonate (MMS) that causes intra-S damage.

Eukaryotic cells respond to replication interference through a complex signal-transduction pathway, known as the S-phase checkpoint, whose key players in the budding yeast *Saccharomyces cerevisiae* are the Mec1 and Rad53 kinases (reviewed in references 5 and 63). Mec1, together with its interacting protein Ddc2, is recruited to stalled forks, where it activates the effector kinase Rad53. Both kinases act in various ways to respond to replication interference. They are needed to complete DNA replication after exposure to HU or MMS (16, 55) by maintaining the integrity and/or activity of the replication forks (11, 15, 26, 34). Furthermore, they stimulate dNTP production (1, 25, 64, 65) and the transcription of several MCB binding factor (MBF)-regulated genes that are involved in DNA replication (2, 58). Finally, they are required for inhibition of late replication origin firing (45, 49) and for preventing accumulation of aberrant DNA structures, such as reversed forks or excessive single-stranded DNA (ssDNA) (20, 33, 50). Despite their inability to replicate DNA, HU-treated *mec1* and *rad53* mutant cells proceed to elongate the mitotic spindle and to partition unreplicated or partially replicated DNA (16, 62). This premature chromosome segregation can be the cause of the extensive chromosomal fragmentation that is observed in mammalian cells lacking the Mec1 ortholog ATR (6, 7, 14), indicating that the S-phase checkpoint ensures that DNA replication is complete before cells divide not only in yeast but also in mammals.

Rad53 and Mec1 kinases are essential for cell viability, but cells lacking either Mec1 or Rad53 can be kept alive by overexpression of the *RNR* genes (16) or by the lack of either the Rnr1 inhibitor Sml1 (64) or the transcriptional repressor of the *RNR* genes Crt1 (25). Because dNTP pools are limiting even during a normal S phase (40), these findings suggest that the essential function of Mec1 and Rad53 is to provide cells with sufficient dNTP levels to support DNA replication. This checkpoint-mediated regulation of dNTP pools is thought to be distinct from the checkpoint-mediated regulation of S-phase progression under replication stress, because *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells lacking the Sml1-mediated inhibition of RNR activity are still extremely sensitive to agents that cause replication stress, such as HU and MMS.

Given the essential function of these checkpoint kinases in mediating the response to replication stress, a fundamental question to be addressed is which a process(es) regulated by the checkpoint is critical for the maintenance of cell viability. A hypomorphic *mec1* mutant (*mec1-100*) (38), which does not block late origin firing in HU but is much less HU sensitive than *mec1*Δ *sml1*Δ cells, argues that regulation of late origin firing plays a relatively minor role in maintaining cell viability after exposure to replication stress (56). Cells lacking Mec1 that are kept viable by *SML1* dele-

Received 16 July 2012 Returned for modification 28 August 2012
Accepted 28 September 2012
Published ahead of print 8 October 2012

Address correspondence to Maria Pia Longhese, mariapia.longhese@unimib.it.
* Present address: Veronica Baldo, Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, California, USA.

Supplemental material for this article may be found at http://mcb.asm.org/.

doi:10.1128/MCB.00956-12

tion have been shown to accumulate chromosome breakages during HU treatment as a consequence of not fully replicated chromosomes being under persistent tension exerted by the mitotic spindle (19). However, inhibiting spindle formation via nocodazole treatment does not improve viability of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells during exposure to HU (16, 55), suggesting that precocious chromosome segregation *per se* is not the reason for the loss of viability of HU-treated *rad53* and *mec1* mutants. This finding has lead to the proposal that the DNA replication defects arising when *mec1* and *rad53* mutants experience replication impediments irreversibly commit cells to death during S phase.

To further investigate the role of Mec1 and Rad53 in maintaining cell viability in the presence of replication stress, we searched for extragenic mutations suppressing the hypersensitivity to HU of *mec1*Δ *sml1*Δ cells. By characterizing one of the identified suppressor mutations, we provide evidence that decreased activity of the cyclin-dependent kinase (Cdk1 or Cdc28 in yeast) complex suppresses *mec1* and *rad53* cell lethality not only during exposure to replication stress but also during an unchallenged S-phase. Delaying either the G_1/S transition or spindle elongation improves viability of HU-treated *mec1* and *rad53* mutants and bypasses the Mec1 and Rad53 essential function during an unperturbed S phase. Further investigation of the suppression mechanism suggests that cell death caused by the lack of the S-phase checkpoint may be a consequence of Cdk1 activity forcing unscheduled events, such as the G_1/S transition and spindle elongation.

MATERIALS AND METHODS

Screening for suppressors of the HU sensitivity of *mec1*Δ *sml1*Δ cells. We searched for spontaneous extragenic mutations suppressing the HU sensitivity of *mec1*Δ *sml1*Δ cells. Since 5 mM HU was the minimal HU dose impairing the ability of *mec1*Δ *sml1*Δ cells to form colonies, we plated *mec1*Δ *sml1*Δ (YLL490) cells on yeast extract-peptone-dextrose (YEPD) plates containing 5 mM HU and searched for clones able to form colonies. This analysis allowed us to identify 20 independent clones able to grow on 5 mM HU. By crossing these clones with a *MEC1 sml1*Δ strain, we found that the suppressor phenotype for two of them was due to a single-gene recessive mutation. One of these two clones was also temperature sensitive for growth, and this phenotype segregated tightly linked to the suppressor phenotype. We cloned the corresponding gene by transforming the original mutant clone with a yeast genomic DNA library constructed in a *LEU2* centromeric plasmid and searching for recombinant plasmids able to inhibit the mutant ability to form colonies on 5 mM HU. Analysis of several positive transformant clones revealed that the minimal complementing region was restricted to a DNA fragment containing the *CDC28* gene. Further genetic analysis allowed us to demonstrate that *CDC28* was indeed the gene identified by the suppressor mutation.

Yeast strains and growth conditions. All yeast strains (see Table S1 in the supplemental material) were derivatives of W303 (*ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, and *rad5-535*). Gene deletions were generated by one-step gene replacement. The *cdc28-as1* mutant, kindly provided by R. Kolodner (San Diego, CA), was backcrossed three times with W303. The *cdc28-1N* mutant and the strain expressing green fluorescent protein (GFP)-tagged Tub1 were kindly provided by Simonetta Piatti (Montpellier, France). The strain expressing both the nucleoside transporter hENT and the herpes simplex virus thymidine kinase used for bromodeoxyuridine (BrdU) incorporation was kindly provided by J. Diffley (South Mimms, United Kingdom). All of the strains expressing the *RAD52-YFP* fusion were derivatives of strain W3749/4C, kindly provided by R. Rothstein (New York, NY). Cells were grown in either synthetic minimal medium supplemented with the appropriate nutrients or YEP (1% yeast extract, 2% Bacto peptone, 50 mg of adenine/liter) medium supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 2% galactose (YEPRG). Benomyl and nocodazole were used at 10 and 5 μg/ml, respectively, in 1% dimethyl sulfoxide. Unless otherwise indicated, the experiments were performed at 25°C.

Microscopy. To visualize the mitotic spindle, cells expressing *TUB1-GFP* were fixed in 100% ethanol at the time points of interest and then washed in 10 mM Tris (pH 8.0) pending microscopic analysis. The GFP fluorophore was visualized using a band-pass GFP filter. To visualize Rad52-YFP foci, cells expressing *RAD52-YFP* were grown in synthetic medium supplemented with adenine to minimize autofluorescence. The cells were washed in 0.1 M potassium phosphate buffer at the time points of interest and analyzed immediately at the microscope. Cells were imaged on concanavalin A-coated slides. Microscopy was performed on a Leica TCS resonant STED DMI6000 CS microscope equipped with a multiline argon ion laser. Images of the YFP-stained yeast cells were acquired by collecting between 530 and 600 nm the fluorescence excited by the 27-μW output of the 514-nm line of the argon laser. Both the emission and the transmitted light images have been recorded at 400-Hz scan speed through a 100× HCX PL APO oil objective (numerical aperture = 1.4) after identification of the cellular focal plane by 1-μm step z-scan measurements. Microscopy images were analyzed by using ImageJ.

Other techniques. Nuclear division was scored with a fluorescence microscope on cells stained with propidium iodide. Flow cytometric DNA analysis was determined on a Becton Dickinson FACScan. The pulse-chase BrdU experiment and immunodetection of BrdU-labeled DNA were performed as described previously (48, 61). For spot assays, exponentially growing overnight cultures were counted, and 10-fold serial dilutions of equivalent cell numbers were spotted onto plates containing the indicated media. Experiments involving G_1 synchronization were carried out by incubating exponentially growing cells in appropriate media containing 5 μg of α-factor/ml at 25°C for 2 h.

RESULTS

Decreased Cdk1 activity improves viability of *mec1* and *rad53* mutants both in the absence and in the presence of replication stress. Budding yeast cells lacking Mec1 or Rad53 and kept viable by *SML1* deletion (*mec1*Δ *sml1*Δ or *rad53*Δ *sml1*Δ) die even when exposed to very low HU doses (Fig. 1A and B). To understand the nature of this lethality, we searched for spontaneous mutations that confer increased HU resistance to *mec1*Δ *sml1*Δ cells (see Materials and Methods). Given the extremely high HU sensitivity of *mec1* mutants, this screening was performed using 5 mM HU, which is the minimal HU dose impairing the ability of *mec1*Δ *sml1*Δ cells to form colonies. One of the suppressors turned out to be a mutation in the *CDC28* gene (see Materials and Methods), which encodes for the catalytic subunit Cdc28/Cdk1 of cyclin-dependent kinase. This mutation (*cdc28-sup*) improved viability of *mec1*Δ *sml1*Δ cells in the presence of either MMS or low HU doses (Fig. 1A). Suppression was not restricted to the *MEC1* deletion, since *cdc28-sup* also decreased the HU and MMS sensitivity of *ddc2*Δ *sml1*Δ cells (Fig. 1A). Cells carrying the *cdc28-sup* mutation were also temperature sensitive for growth, but they did not show a uniform terminal phenotype when shifted to 37°C (data not shown).

Mec1 might function in supporting cell viability in the presence of HU because it is required to activate the downstream kinase Rad53. However, *mec1*Δ *sml1*Δ cells are considerably more sensitive to HU and other DNA-damaging agents than are *rad53*Δ *sml1*Δ cells (16), suggesting that Mec1 and Rad53 might have different roles during DNA replication under stress conditions. Thus, we sought to determine whether the *cdc28-sup* mutation could suppress also the HU sensitivity of *rad53*Δ *sml1*Δ or *rad53-K227A* mutant cells, the latter expressing a Rad53 mutant variant with reduced kinase activity that supports cell viability even in the presence of Sml1 (53). The ability of *rad53*Δ *sml1*Δ *cdc28-sup* and

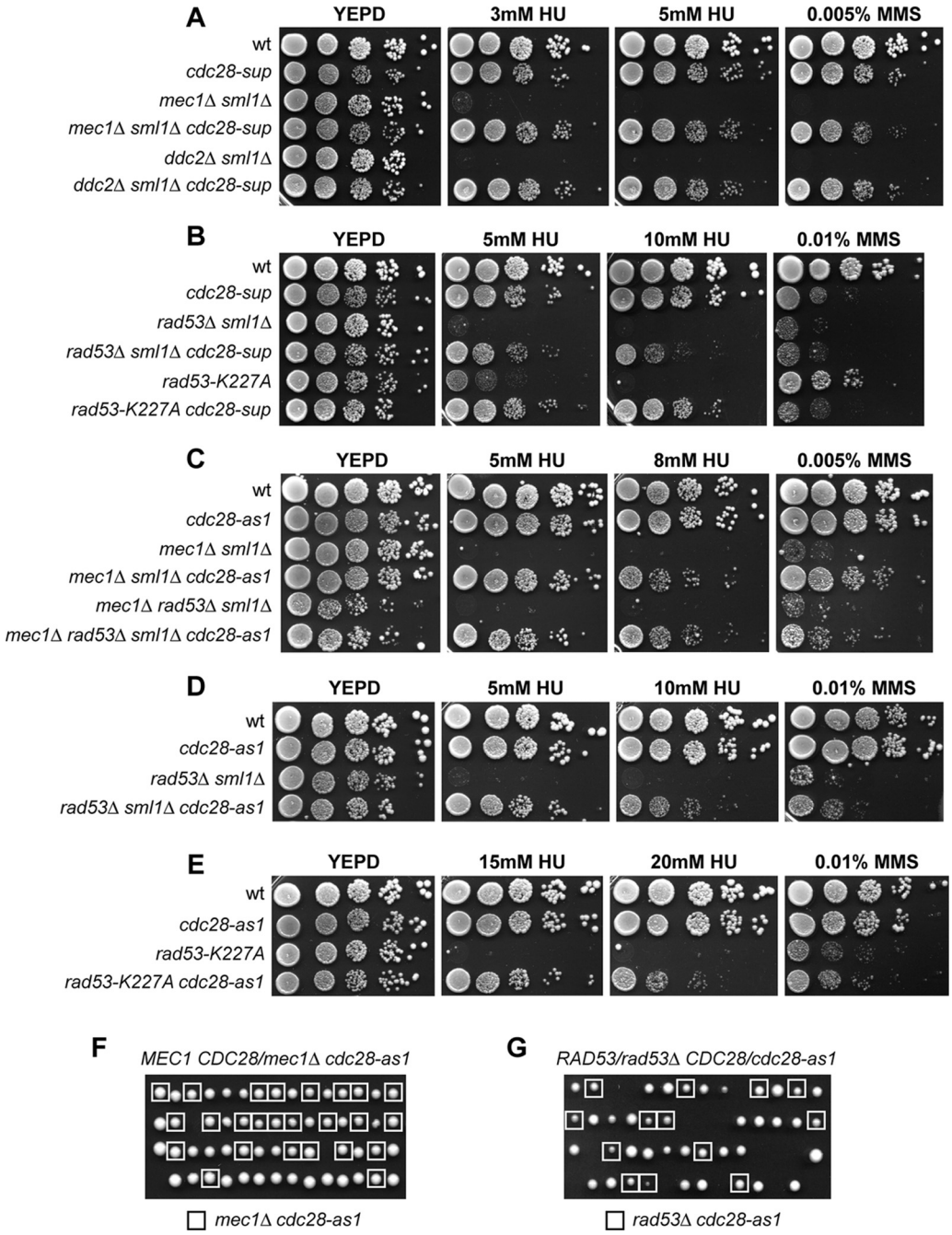

FIG 1 Hypomorphic mutations in *CDC28* improve viability of *mec1* and *rad53* mutants. (A to E) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3 days. (F and G) Meiotic tetrads from *MEC1 CDC28*/*mec1*Δ *cdc28-as1* and *RAD53*/*rad53*Δ *CDC28*/*cdc28-as1* diploid strains were dissected on YEPD plates, which were incubated at 25°C for 3 days. Clones from double-mutant spores are highlighted by squares.

rad53-K227A cdc28-sup cells to form colonies in the presence of HU was higher than that of *rad53*Δ *sml1*Δ and *rad53-K227A* cells, respectively, indicating that *cdc28-sup* can suppress also the HU sensitivity caused by Rad53 dysfunction (Fig. 1B). We were unable to determine whether *cdc28-sup* suppressed the MMS sensitivity of *rad53* mutants because the *cdc28-sup* mutation by itself caused cell lethality in the presence of the amount of MMS that was required to impair viability of *rad53* mutants (Fig. 1B).

To assess whether the Cdk1-dependent lethality in HU of *mec1* and *rad53* cells could be attributed to Cdk1 kinase activity, we constructed *mec1* and *rad53* mutants expressing the *cdc28-as1* allele, which encodes for a kinase with an enlarged ATP-binding pocket, allowing it to bind the nonhydrolyzable ATP analogue 1-NM-PP1. Treatment of *cdc28-as1* cells with 1-NM-PP1 results in rapid downregulation of Cdc28 kinase activity, but Cdc28-as1 kinase activity is reduced by ~20% compared to wild type even in

the absence of 1-NM-PP1 (4). We found that survival to HU of *mec1*Δ *sml1*Δ *cdc28-as1* cells was higher than that of *mec1*Δ *sml1*Δ cells even in the absence of 1-NM-PP1 (Fig. 1C). Furthermore, the *cdc28-as1* mutation diminished the HU sensitivity of both *rad53*Δ *sml1*Δ (Fig. 1D) and *rad53-K227A* cells (Fig. 1E). Therefore, a reduced Cdk1 activity counteracts cell death in *mec1* and *rad53* mutants exposed to replication impediments. The HU sensitivity of *mec1*Δ *rad53*Δ *sml1*Δ cells was similar to that of *mec1*Δ *sml1*Δ cells (Fig. 1C), and *cdc28-as1* improved survival in response to HU treatment of *mec1*Δ *sml1*Δ and *mec1*Δ *rad53*Δ *sml1*Δ cells to the same extent (Fig. 1C). Thus, suppression in *mec1*Δ *sml1*Δ mutant does not appear to require the activity of Rad53.

Interestingly, although *mec1*Δ *sml1*Δ cells were more sensitive to MMS than *rad53*Δ *sml1*Δ cells (compare Fig. 1C and D), the *cdc28-as1* mutation suppressed the sensitivity to MMS of *mec1*Δ *sml1*Δ more efficiently to that of both *rad53*Δ *sml1*Δ and *rad53-K227A* cells (Fig. 1C to E). These findings suggest that the causes of death in MMS-treated *mec1* and *rad53* mutants are different, supporting previous data showing that Mec1 and Rad53 play different roles in the response to MMS treatment (48). Although the MMS sensitivity of *mec1*Δ *rad53*Δ *sml1*Δ cells was similar to that of *mec1*Δ *sml1*Δ cells (Fig. 1C), *cdc28-as1* improved survival in response to MMS treatment of *mec1*Δ *sml1*Δ cells more efficiently than that of *mec1*Δ *rad53*Δ *sml1*Δ cells (Fig. 1C and D), indicating that Rad53 contributes to the *cdc28-as1*-mediated suppression of the MMS sensitivity caused by the lack of Mec1.

Rad53 and Mec1 are essential for cell viability, prompting us to ask whether a reduced Cdk1 activity could also bypass the essential function of these checkpoint kinases. Diploid strains heterozygous for *cdc28-as1* and either *mec1*Δ or *rad53*Δ alleles were generated and spore viability was monitored after tetrad dissection. Since the *MEC1* and *CDC28* genes are linked to each other on chromosome II, *mec1*Δ and *cdc28-as1* alleles were expected to cosegregate in most tetrads from the *MEC1 CDC28/mec1*Δ *cdc28-as1* diploid. As expected, *mec1*Δ and *rad53*Δ spores failed to form colonies, whereas all of the *mec1*Δ *cdc28-as1* (Fig. 1F) and *rad53*Δ *cdc28-as1* (Fig. 1G) double-mutant spores formed colonies of almost wild-type size, indicating that a reduced Cdk1 activity rescues the lethality caused by the lack of either Mec1 or Rad53. Collectively, these data indicate that carrying out a certain Cdk1-dependent event(s) causes cell death in the absence of Mec1 or Rad53 independently of exogenous DNA replication stress.

Decreased Cdk1 activity suppresses the DNA replication defects of *mec1* and *rad53* mutants exposed to replication stress. To understand the molecular mechanisms underlying the suppression described above, we analyzed the effects of the *cdc28-as1* mutation on the kinetics of DNA replication and cell cycle progression of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells exposed to a low HU dose. Cell cultures were blocked in G_1 with α-factor and released from the G_1 arrest either in the absence or in the presence of 20 mM HU (Fig. 2). As expected (16), *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells released in the presence of HU were unable to complete DNA replication even at 360 min after α-factor release (Fig. 2A). However, most cells partitioned incompletely replicated DNA, undergoing nuclear division at 90 min after the release (Fig. 2C). Consistent with the requirement of Cdk1 to perform the G_1/S and G_2/M transitions, the presence of *cdc28-as1* delayed bud formation (Fig. 2B) and nuclear division (Fig. 2C) of wild-type, *mec1*Δ *sml1*Δ, and *rad53*Δ *sml1*Δ cells released in the presence of HU. Then, HU-treated *mec1*Δ *sml1*Δ *cdc28-as1* and *rad53*Δ *sml1*Δ *cdc28-as1* cells exited from mitosis, divided, and initiated a new cell cycle, whereas similarly treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells arrested as budded cells with two nuclei, as expected (Fig. 2A to C). Accordingly, the survival rates in HU of *mec1*Δ *sml1*Δ *cdc28-as1* and *rad53*Δ *sml1*Δ *cdc28-as1* were higher than those of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells throughout the experiment (Fig. 2D). Thus, reducing Cdk1 activity in HU-treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells restores cell cycle progression and cell viability.

Interestingly, *mec1*Δ *sml1*Δ *cdc28-as1* and *rad53*Δ *sml1*Δ *cdc28-as1* cells seemed to have completed the bulk DNA synthesis at the time of nuclear division (Fig. 2A and C), suggesting that lowering the Cdk1 activity suppresses the DNA replication defects of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells. To follow more directly the DNA replication kinetics, we performed BrdU pulse-chase experiments. Cells were synchronized in G_1 with α-factor and released into medium containing 20 mM HU and BrdU for 15 min (Fig. 2E) to label the nascent DNA. The BrdU was then chased by transferring cells to medium containing thymidine at a high concentration and 20 mM HU (Fig. 2E). Labeled nascent DNA replication intermediates, which appeared as a smear in all of the strains after 15 min in HU plus BrdU, rapidly increased in size after the chase in wild-type cells (Fig. 2F). Consistent with a failure of *mec1* and *rad53* mutants to complete DNA replication, the formation of high-molecular-weight molecules of nascent DNA was delayed in *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells. Strikingly, almost all of the incorporated BrdU in *mec1*Δ *sml1*Δ *cdc28-as1* and *rad53*Δ *sml1*Δ *cdc28-as1* cells was present in the high-molecular-weight fraction by 90 to 120 min after α-factor release (Fig. 2F). Altogether, these data suggest that reducing Cdk1 activity increases survival of HU-treated *mec1* and *rad53* mutants by suppressing their DNA replication defects.

Cdk1-dependent lethality of *mec1* and *rad53* mutants involves a Cdk1 function at the G_1/S transition. Regulation of Cdk1 activity during the cell cycle depends on the interaction of the Cdk1 kinase with different cyclin subunits. In budding yeast, the partially redundant G_1 cyclins Cln1 to Cln3 are required to perform the G_1/S transition (43). Interestingly, deletion of both *CLN1* and *CLN2* was shown to partially bypass the essential requirement of Mec1, whereas overexpression of *CLN1* or *CLN2* (but not of *CLN3*) exacerbated the growth defects of *mec1* mutants in the absence of genotoxic agents (59). We therefore sought to determine whether the Cdk1-dependent lethality of HU-treated *mec1* and *rad53* cells could be attributed to a function of Cdk1 in G_1. Deletion of *CLN2* improved the viability of *mec1*Δ *sml1*Δ, *rad53*Δ *sml1*Δ, and *rad53-K227A* mutant cells exposed to moderate HU doses (Fig. 3A), whereas *CLN1* deletion did not (data not shown). Similar to what we observed for the *cdc28-as1* allele, the lack of Cln2 suppressed the MMS sensitivity of *mec1*Δ *sml1*Δ cells, but only a very weak (if any) suppressor effect was detectable in *rad53*Δ *sml1*Δ and *rad53-K227A* cells (Fig. 3A).

We also found that the lack of Cln2 bypasses the essential function of Mec1 and Rad53. In fact, tetrad dissection of diploid strains heterozygous for *cln2*Δ and *mec1*Δ alleles showed that all of the *mec1*Δ *cln2*Δ double-mutant spores formed colonies, although with a smaller size than wild-type or *cln2*Δ spores (Fig. 3B). Similar results were obtained by combining the *cln2*Δ and *rad53*Δ alleles (data not shown).

The function of Cln1 and Cln2 in G_1 is to promote entry into S-phase by activating the S-phase Clb5-6/Cdk1 complexes, which in turn trigger replication origin firing. This Cln/Cdk1 function is

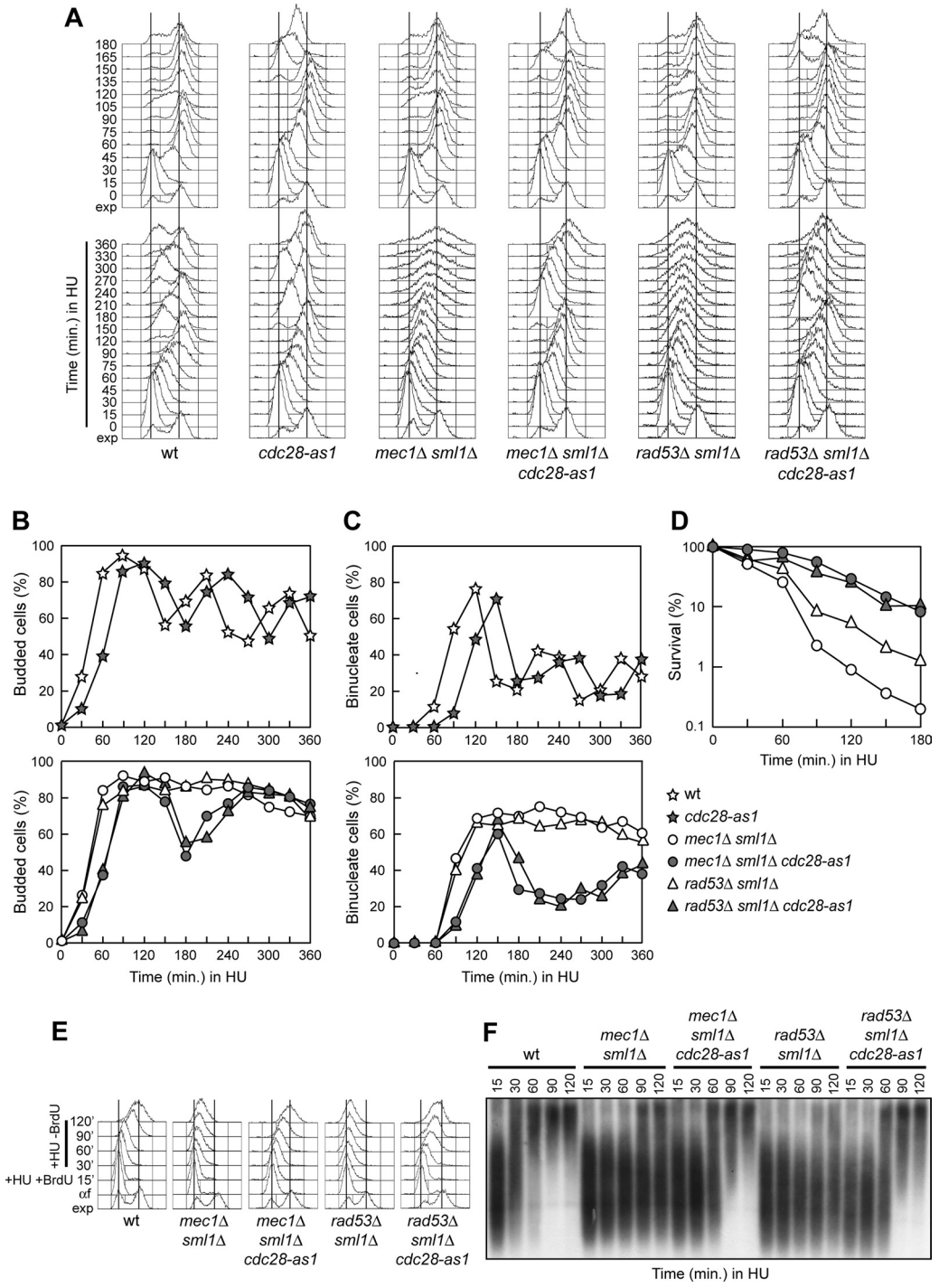

FIG 2 Effects of *cdc28-as1* on DNA replication and cell cycle progression of HU-treated *mec1* and *rad53* cells. (A to D) Exponentially growing cultures of cells with the indicated genotypes were arrested in G_1 with α-factor and released at 25°C in YEPD in the absence or in the presence of 20 mM HU. At the indicated times after α-factor release, cell samples were taken for fluorescence-activated cell sorting (FACS) analysis of DNA content (A) and for scoring budding (B) and nuclear division (C). (D) Cell survival in the liquid cultures described in panels A to C was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after α-factor release, followed by incubation at 25°C for 3 days. (E and F) G_1-arrested cells (αf) were released into YEPRG containing 20 mM HU plus 25 μM BrdU. After 15 min (+HU +BrdU 15′), the cells were chased with 2 mM thymidine into fresh medium containing 20 mM HU (+HU −BrdU). Cell samples were taken at the indicated times for FACS analysis of DNA content (E) and for detecting BrdU-labeled DNA with anti-BrdU antibodies after gel electrophoresis and transfer to a membrane (F).

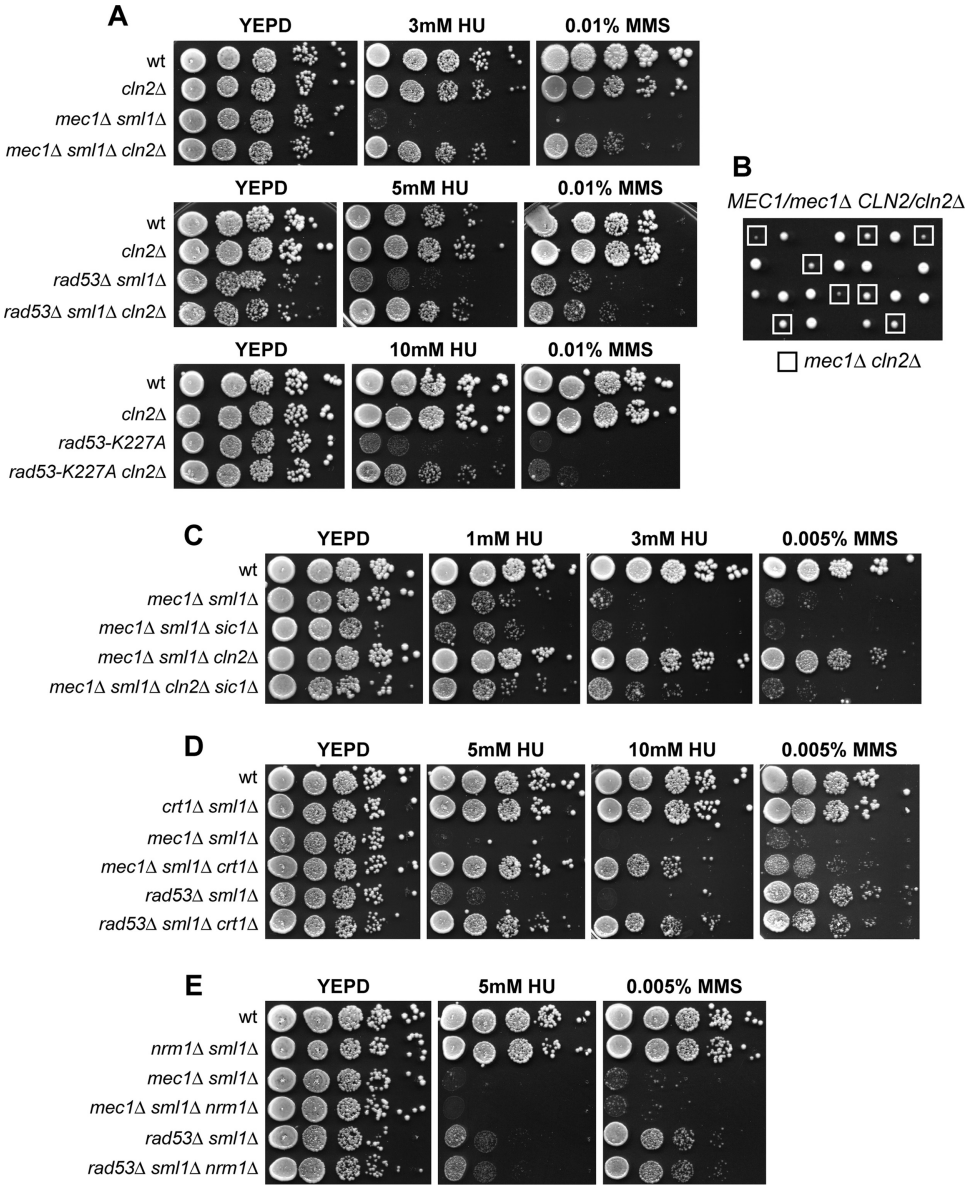

FIG 3 The lack of Cln2 improves viability of *mec1* and *rad53* mutants. (A and C to E) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3 days. (B) Meiotic tetrads from a *MEC1*/*mec1*Δ *CLN2*/*cln2*Δ diploid strain were dissected on YEPD plates, which were incubated for 3 days at 25°C. Clones from double-mutant spores are highlighted by squares.

accomplished through phosphorylation and degradation of the specific Clb/Cdk1 inhibitor Sic1 (47, 60). At the G_1/S transition, the S-phase checkpoint induces transcription of the *RNR* genes and of several MBF-regulated genes through the inhibition of transcriptional repressors Crt1 (25) and Nrm1 (2, 58), respectively. Whether this checkpoint-mediated transcriptional program helps cell survival in the presence of replication stress is not known. Because *CLN2* deletion causes a G_1/S transition delay (17), one possibility is that this delay provides *mec1* and *rad53* mutant cells with a longer time to amend the amount of dNTPs and/or proteins that are required to support DNA replication under stress conditions. If this were the case, the *cln2*Δ-mediated suppression should be overcome by abrogating the G_1/S delay. Indeed, deletion of *SIC1*, which is known to abolish the delay in S-phase entry caused by the absence of Cln2 (17), counteracted the ability of *cln2*Δ to suppress the HU and MMS sensitivity of *mec1*Δ *sml1*Δ cells. In fact, loss of viability of *mec1*Δ *sml1*Δ *cln2*Δ *sic1*Δ mutant cells after plating on HU- and MMS-containing media was similar to that of *mec1*Δ *sml1*Δ *sic1*Δ and *mec1*Δ *sml1*Δ cells (Fig. 3C). This finding suggests that *CLN2* deletion can suppress the sensitivity to replication stress of *mec1* and *rad53* mutant cells by delaying entry into S phase.

The data presented above imply that the role of Mec1 and Rad53 in inducing transcription is important to maintain cell survival in the presence of replication stress. To further assess this possibility, we sought to determine whether the lack of the transcriptional repressor Crt1 or Nrm1 improves the survival in response to HU and/or MMS of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells. Indeed, the lack of Crt1, which was previously shown to bypass the essential function of Mec1 (25), decreased the HU and

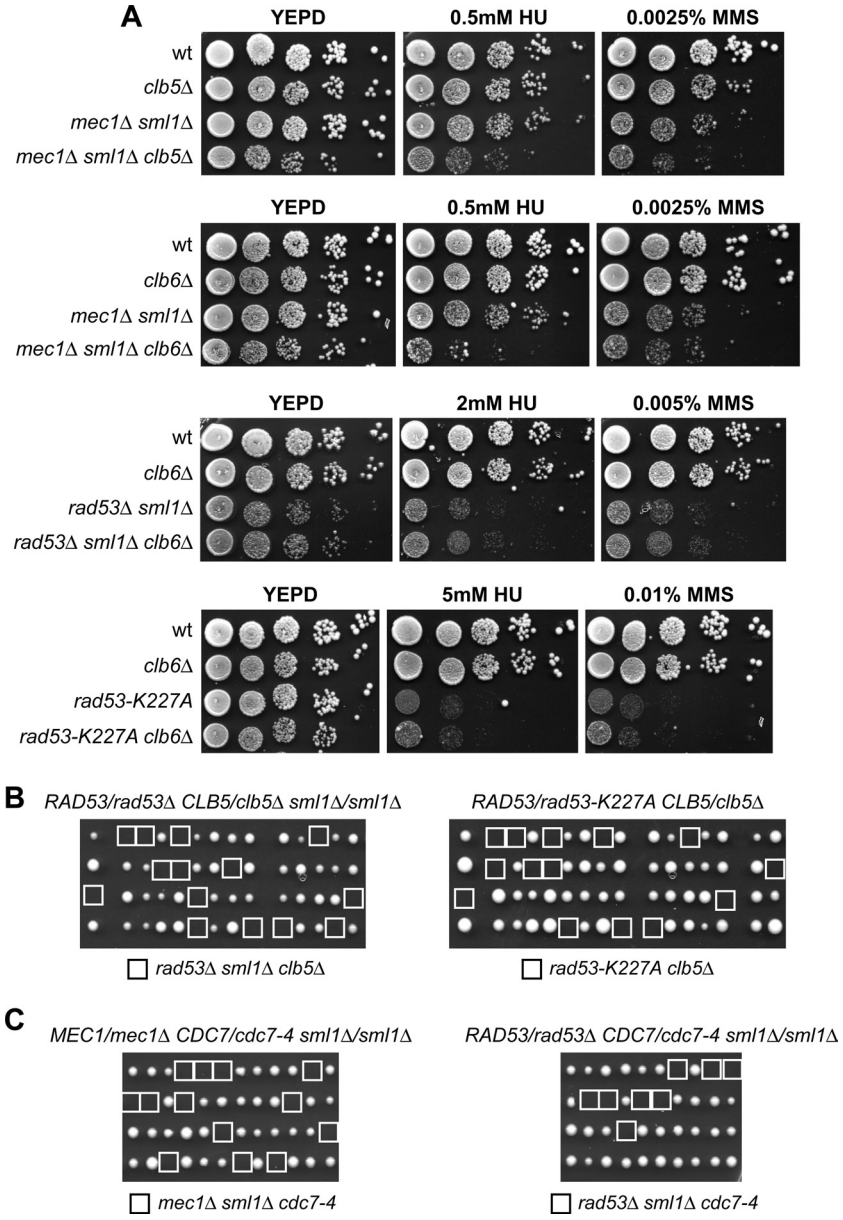

FIG 4 *CLB5* deletion affects viability of *mec1* and *rad53* mutants. (A) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3 days. (B) Meiotic tetrads from *RAD53*/*rad53*Δ *CLB5*/*clb5*Δ *sml1*Δ/*sml1*Δ and *RAD53*/*rad53-K227A CLB5*/*clb5*Δ diploid strains were dissected on YEPD plates, which were incubated for 3 days at 25°C. (C) Meiotic tetrads from *MEC1*/*mec1*Δ *CDC7*/*cdc7-4 sml1*Δ/*sml1*Δ and *RAD53*/*rad53*Δ *CDC7*/*cdc7-4 sml1*Δ/*sml1*Δ diploid strains were dissected on YEPD plates, which were incubated for 3 days at 25°C. The squares in panels B and C highlight putative double-mutant spores based on viable spore genotyping.

MMS sensitivity of *mec1*Δ *sml1*Δ cells and the HU sensitivity of *rad53*Δ *sml1*Δ cells (Fig. 3D), whereas the lack of Nrm1 did not (Fig. 3E). Thus, checkpoint-mediated inhibition of Crt1 and subsequent transcriptional activation of the *RNR* genes help cells to survive in the presence of replication stress.

Decreasing S-phase Cdk1 function is detrimental in *mec1* and *rad53* mutants. In *S. cerevisiae*, the two B-type cyclins—Clb5 and Clb6—stimulate the function of Cdk1 in promoting replication origin firing, with Clb5 playing the major role (18, 28, 46). Decreasing Clb5/Clb6-Cdk1 activity might improve the viability of HU- and/or MMS-treated *mec1* and *rad53* mutants by lowering the total number of replication forks and therefore the chance to block them. This does not seem to be the case, since neither *CLB5* deletion nor *CLB6* deletion suppressed the sensitivity to HU or MMS of *mec1* and *rad53* cells (Fig. 4A). On the contrary, *mec1*Δ *sml1*Δ *clb5*Δ and *mec1*Δ *sml1*Δ *clb6*Δ cells displayed enhanced sensitivity to HU and MMS compared to each single mutant (Fig. 4A). Moreover, consistent with previous data (21), *mec1*Δ *sml1*Δ *clb5*Δ cells grew less efficiently than *mec1*Δ *sml1*Δ cells even in the absence of HU or MMS (Fig. 4A), and the deletion of *CLB5* was lethal in both *rad53*Δ *sml1*Δ and *rad53-K227A* mutant cells (Fig. 4B).

Loss of viability of *rad53 clb5*Δ and *mec1 clb5*Δ double-mutant

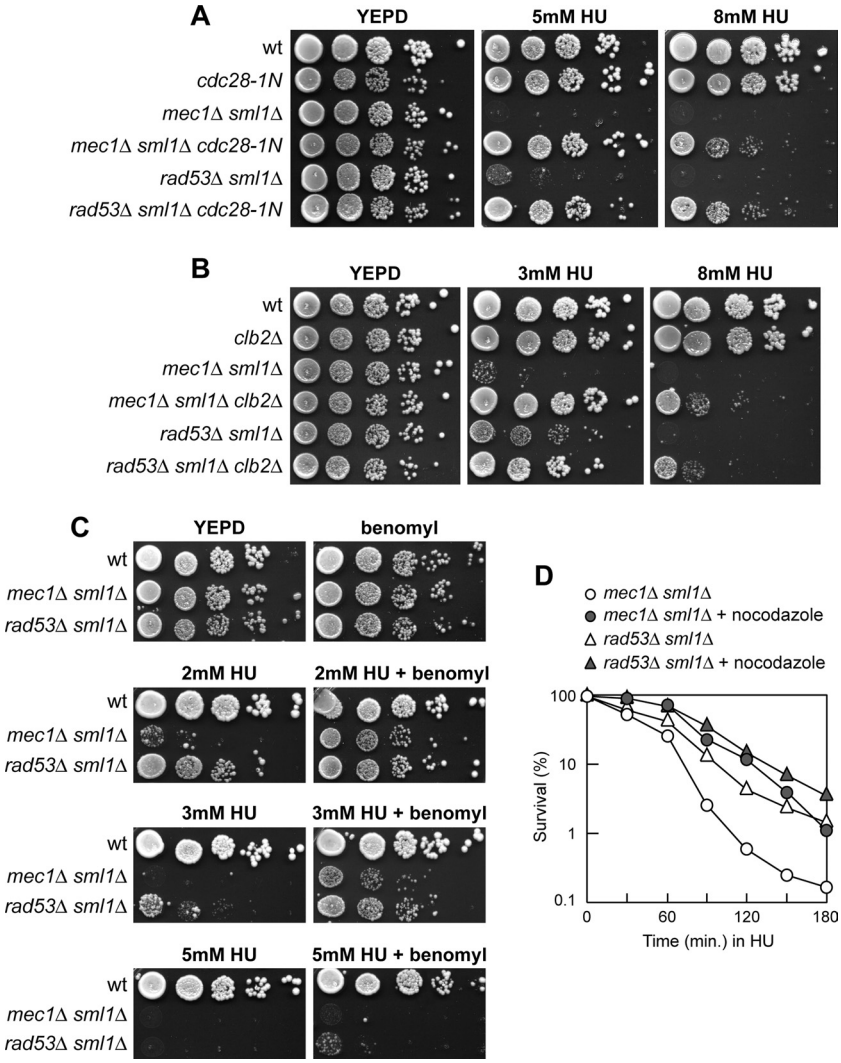

FIG 5 Delaying spindle formation improves the viability of *mec1* and *rad53* mutants during replicative stress. (A and B) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days. (C) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates containing the indicated HU concentrations in the presence or absence of 10 μg of benomyl/ml. (D) G_1-synchronized cultures were divided in two, and one-half was released into YEPD containing 20 mM HU and 5 μg of nocodazole/ml, whereas the other half was released into YEPD containing only 20 mM HU. Cell survival was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after α-factor release, followed by incubation at 25°C for 3 days. The experiment was repeated three times with similar results.

cells even in the absence of the Sml1-mediated inhibition of RNR activity might be due to a diminished number of fired replication origins and therefore of active replication forks that can complete DNA replication before cells divide. Since origin firing throughout S-phase requires also the Cdc7-Dbf4 (DDK) complex (reviewed in reference 29), we analyzed the consequences of introducing the *cdc7-4* allele in *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ mutants. No viable *rad53*Δ *sml1*Δ *cdc7-4* and *mec1*Δ *sml1*Δ *cdc7-4* spores could be found after tetrad dissection of homozygous *sml1*Δ diploid strains, which were heterozygous for *cdc7-4* and either *mec1*Δ or *rad53*Δ, whereas each kind of single mutant spores was obtained at normal frequency (Fig. 4C).

Thus, a decreased S-phase function of Cdk1 cannot account for the suppressor effect on the HU sensitivity of *mec1* and *rad53* mutants observed by reducing Cdk1 activity. Instead, disabling S-phase Cdk1 complexes impairs viability of these mutants even in the absence of exogenous replication impediments, possibly because it diminishes the number of active replication forks and therefore the chance to complete DNA replication.

Cdk1-dependent lethality of *mec1* and *rad53* mutants involves a G_2/M function of Cdk1. To investigate whether the Cdk1-dependent lethality of *mec1* and *rad53* cells could implicate also some mitotic Cdk1 functions, we sought to determine whether the *cdc28-1N* allele, which is specifically defective in the interaction with the mitotic cyclins (54), could suppress the HU and MMS sensitivity of *mec1* and *rad53* mutants. Furthermore, because Cdk1 association with the mitotic cyclins Clb1 to Clb4 drives spindle assembly and progression to metaphase (41), we also analyzed the consequences of deleting the mitotic cyclin gene *CLB2*. The *cdc28-1N* allele increased survival to HU of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells (Fig. 5A). Furthermore, viability on HU of *mec1*Δ *sml1*Δ *clb2*Δ and *rad53*Δ *sml1*Δ *clb2*Δ cells was

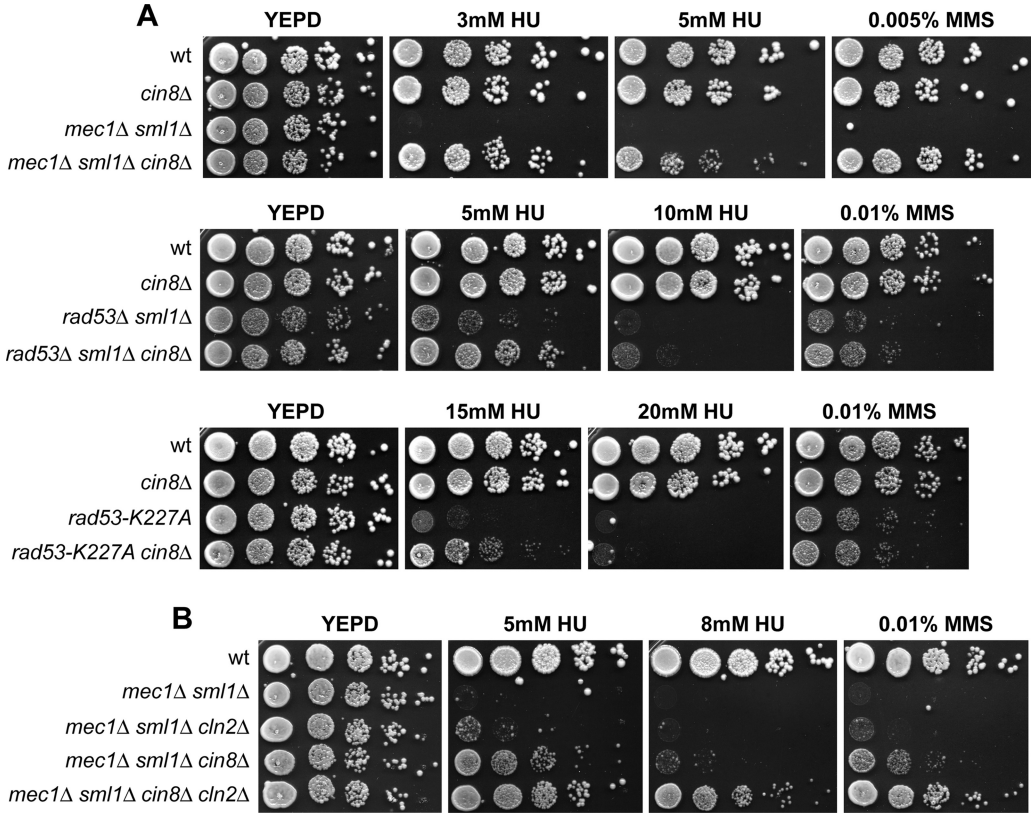

FIG 6 The lack of Cin8 increases survival of *mec1* and *rad53* mutants under replicative stress. Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3 days.

higher than that of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells, respectively (Fig. 5B). Thus, a mitotic function of Cdk1 contributes to impair viability of HU-treated *mec1* and *rad53* mutants.

Although *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells are unable to complete DNA replication in the presence of HU, they prematurely elongate the spindles and partition unreplicated or partially replicated chromosomes (16, 62). Since Cdk1 activity is required to assemble a mitotic spindle (41), decreased Cdk1 activity might suppress the sensitivity of *mec1* and *rad53* mutants to replication stress by providing the cells with a longer time to finish replicating the bulk of their chromosomes before they segregate. If this were the case, cell death in these mutants should be suppressed by disrupting kinetochore-microtubule attachment via nocodazole or benomyl treatment. Indeed, benomyl addition to HU-containing medium improved the viability of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells (Fig. 5C). Furthermore, the survival rates of G_1-arrested *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells after release into YEPD medium containing 20 mM HU and nocodazole were higher than those of the same cells released into YEPD containing only 20 mM HU (Fig. 5D). Interestingly, nocodazole addition suppressed the HU sensitivity of *mec1*Δ *sml1*Δ cells more efficiently than that of *rad53*Δ *sml1*Δ cells (Fig. 5D), suggesting that the DNA replication defects in HU-treated *mec1* and *rad53* mutants do not completely overlap.

Delaying spindle elongation suppresses the sensitivity of *mec1* and *rad53* mutants to replication stress. Premature spindle elongation and DNA partitioning in HU-treated *mec1* and *rad53* mutants is due to upregulation of the major kinesin motor protein Cin8 (27), which is known to contribute to the formation, stability and extension of the spindles (24, 44, 52). Since Cdk1 activity drives the assembly of mitotic spindles by restraining Cin8 proteolysis (13), delaying spindle extension by deleting *CIN8* might be expected to improve the survival of *mec1* and *rad53* mutants to HU and/or MMS treatment. Indeed, the viability of *mec1*Δ *sml1*Δ *cin8*Δ, *rad53*Δ *sml1*Δ *cin8*Δ, and *rad53-K227A cin8*Δ cells in the presence of HU was considerably higher than that of *mec1*Δ *sml1*Δ, *rad53*Δ *sml1*Δ, and *rad53-K227A* cells, respectively (Fig. 6A), suggesting that premature spindle elongation contributes to kill HU-treated *mec1* and *rad53* mutants. Similar to *cdc28-as1* and *cln2*Δ, *cin8*Δ efficiently suppressed the sensitivity to MMS of *mec1*Δ *sml1*Δ cells but suppressed very poorly that of *rad53*Δ *sml1*Δ cells (Fig. 6A). Moreover, inactivation of both Cin8 and Cln2 suppressed the sensitivity to HU and MMS of *mec1*Δ *sml1*Δ cells more efficiently than did the single Cin8 or Cln2 inactivation (Fig. 6B), in agreement with the notion that both the G_1/S and the G_2/M functions of Cdk1 are involved in determining this sensitivity.

To characterize the *cin8*Δ-mediated suppression, cells were arrested in G_1 with α-factor and released in YEPD in the presence or absence of 20 mM HU. As expected, although HU-treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ mutants failed to complete DNA replication (Fig. 7A), a significant proportion of them elongated the spindles (Fig. 7C), divided the nuclei (Fig. 7D), and then arrested as binucleate cells with elongated spindles. Deletion of *CIN8* did

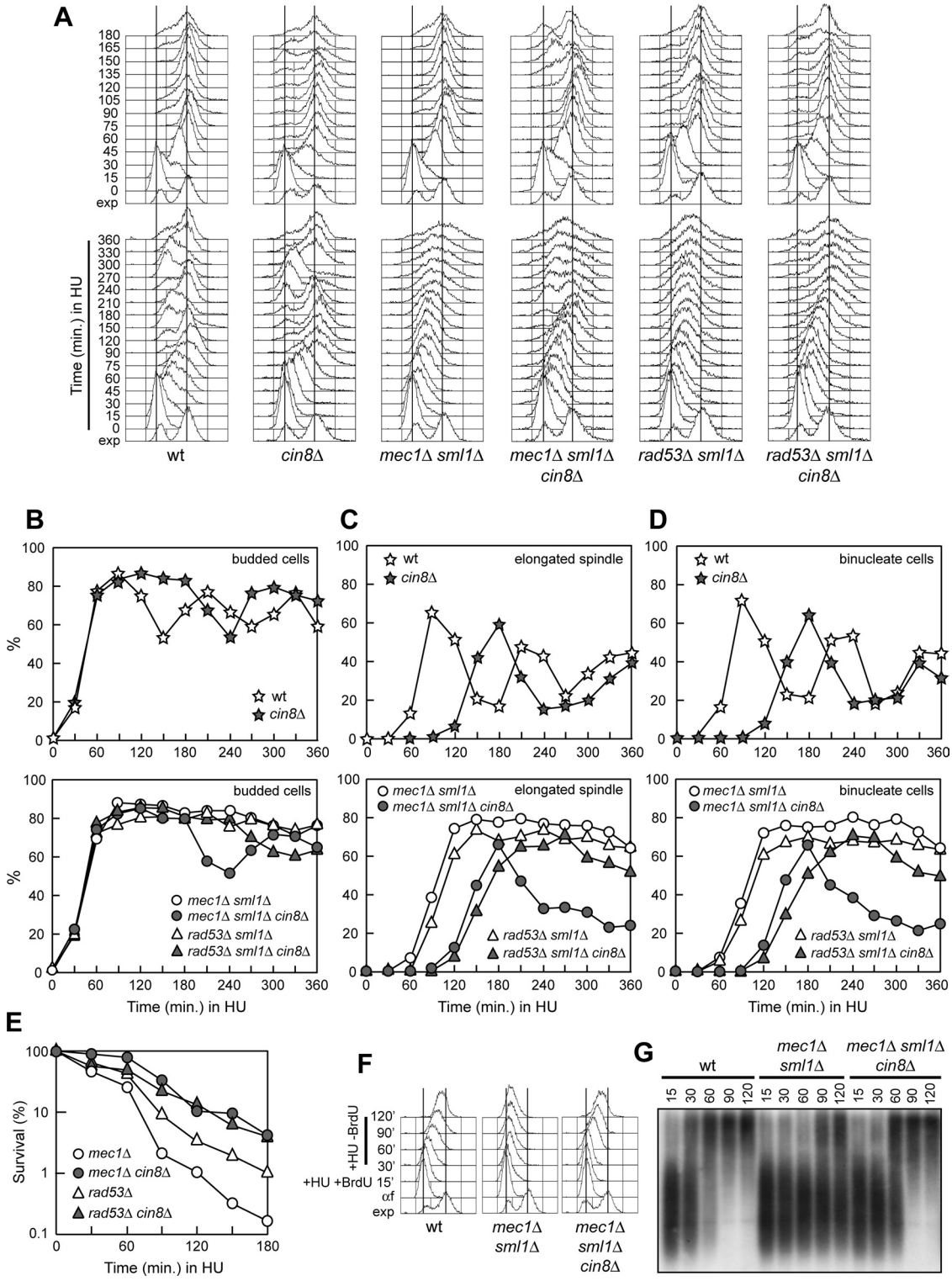

FIG 7 Effect of *CIN8* deletion on cell cycle progression of HU-treated *mec1* and *rad53* mutant cells. (A to E) Exponentially growing cultures of cells with the indicated genotypes, all expressing a Tub1-GFP fusion, were arrested in G_1 with α-factor and released at 25°C in YEPD in the presence or absence of 20 mM HU. Cell samples were taken at the indicated times after α-factor release for FACS analysis of DNA content (A) and for scoring budding (B), spindle elongation (C), and nuclear division (D). (E) Cell survival of the liquid cultures described in panels A to D was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after α-factor release, followed by incubation at 25°C for 3 days. (F and G) Immunodetection of BrdU-pulsed DNA. G_1-arrested cells (αf) were released into YEPRG containing 20 mM HU plus 25 μM BrdU. After 15 min (+HU +BrdU 15′), the cells were chased with 2 mM thymidine into medium containing 20 mM HU (+HU −BrdU). Cell samples were taken at the indicated times after chase for FACS analysis of DNA content (F) and for detecting BrdU-labeled DNA with anti-BrdU antibodies as in Fig. 2F (G).

not affect bud emergence (Fig. 7B) but slowed down spindle elongation and nuclear division in both *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells (Fig. 7C and D). Moreover, the survival rates of HU-treated *mec1*Δ *sml1*Δ *cin8*Δ and *rad53*Δ *sml1*Δ *cin8*Δ were higher than those of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells throughout the experiment (Fig. 7E). Consistent with the finding that *CIN8* deletion (as well as nocodazole addition) suppresses the HU sensitivity of *mec1*Δ *sml1*Δ more efficiently than that of *rad53*Δ *sml1*Δ cells (Fig. 7E), most HU-treated *mec1*Δ *sml1*Δ *cin8*Δ cells exited from mitosis and resumed cell cycle progression within 210 min after release from G_1 arrest, whereas most *rad53*Δ *sml1*Δ *cin8*Δ cells were still arrested after 360 min (Fig. 7A to D).

Interestingly, while spindle elongation and nuclear division in HU-treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells took place when DNA was not fully replicated, the bulk of DNA synthesis seemed to be completed at the time of spindle elongation/nuclear division in *mec1*Δ *sml1*Δ *cin8*Δ and *rad53*Δ *sml1*Δ *cin8*Δ cells under the same conditions (Fig. 7A to D). This indication was confirmed for *mec1*Δ *sml1*Δ *cin8*Δ cells by BrdU pulse-chase experiments during HU exposure. The cells were synchronized in G_1 with α-factor and released into medium containing 20 mM HU and BrdU for 15 min (Fig. 7F) to label the nascent DNA. The BrdU was then chased by transferring cells to medium containing thymidine at a high concentration and 20 mM HU (Fig. 7F). As expected, the formation of high-molecular-weight molecules of nascent DNA was delayed in *mec1*Δ *sml1*Δ cells compared to wild-type cells (Fig. 7G), whereas *mec1*Δ *sml1*Δ *cin8*Δ cells contained almost all of the incorporated BrdU into the high-molecular-weight fraction by 90 to 120 min after α-factor release (Fig. 7G). Collectively, these data indicate that delaying spindle elongation suppresses the DNA replication defects of *mec1* and *rad53* mutants exposed to a mild HU dose, possibly by providing additional time for completing DNA synthesis before spindle elongation and chromosome segregation take place.

Delaying spindle elongation bypasses the essential function of Mec1 and Rad53. Cells lacking Mec1 or Rad53, but carrying wild-type *SML1*, die even in the absence of exogenous replication stress because they replicate their DNA with suboptimal dNTP levels (64). As HU depletes the dNTP pools, the cause of death in *mec1*Δ *SML1* and *rad53*Δ *SML1* cells might mimic that of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells replicating their DNA in the presence of HU. Thus, we sought to determine whether delaying nuclear division by *CLB2* or *CIN8* deletion could bypass the essential function of Mec1 and Rad53. Tetrad dissection of diploid strains heterozygous for *cin8*Δ and either *mec1*Δ or *rad53*Δ alleles showed that most *mec1*Δ *cin8*Δ and *rad53*Δ *cin8*Δ double-mutant spores formed colonies, although with a smaller size than wild-type or *cin8*Δ spores (Fig. 8A). Similar results were obtained by combining the *mec1*Δ or *rad53*Δ mutation with the *clb2*Δ mutation (Fig. 8B). Thus, delaying spindle elongation not only suppresses the sensitivity of *mec1* and *rad53* to replication stress but also bypasses their essential function, suggesting that loss of viability in *mec1*Δ *SML1* and *rad53*Δ *SML1* cells is at least partially due to segregation of incompletely replicated chromosomes.

Segregation of partially replicated chromosomes may contribute to cell death because it causes lethal DNA breaks. Since DNA breaks are subjected to the action of the recombination protein Rad52 (30), we investigated whether conditional inactivation of Mec1 in the presence of wild-type *SML1* leads to the formation of Rad52 foci. We failed to efficiently deplete Mec1 by using repressible promoters or inducible degradation systems, and therefore we conditionally inactivated Mec1 by using the temperature-sensitive *mec1-14* allele that we identified previously (32). Consistent with our finding that the *cdc28-as1* and *clb2*Δ alleles bypass the essential function of Mec1, both the *cdc28-as1* and *clb2*Δ mutations improved the viability of *mec1-14* cells at 34°C (Fig. 8C). When wild-type and *mec1-14* cell cultures were arrested in G_1 with α-factor at 25°C and released into the cell cycle at 37°C, both cell types showed similar kinetics of bud emergence (Fig. 8D). However, *mec1-14* cells delayed completion of DNA replication compared to wild-type cells, indicating a DNA replication defect (Fig. 8E). Moreover, *mec1-14* cells dramatically accumulated Rad52 foci about 90 min after α-factor release at 37°C, concomitantly with nuclear division (Fig. 8F and G), suggesting that lethal chromosome breaks occur when *mec1-14* cells enter mitosis. These Rad52 foci could arise as a consequence of the action of endonucleases, which process the replication intermediates that persist until mitosis in *mec1-14* cells. Alternatively, segregation of incompletely replicated chromosomes can be the cause of Rad52 focus formation in Mec1-deficient cells. Since the endonucleases Mms4 and Yen1, which are known to resolve recombination intermediates, are activated at the G_2/M transition (35), we analyzed their contribution in the generation of Rad52 foci in *mec1-14* cells. We found that the lack of Mms4, Yen1, or both did not impair Rad52 focus formation in *mec1-14* cells (Fig. 8H). Rather, the lack of both Mms4 and Yen1 caused an increase of Rad52 foci in *mec1-14* cells even at 25°C (time zero in Fig. 8H), possibly because these two nucleases are involved in repairing the double-strand breaks that arise in *mec1-14* cells. Interestingly, when *mec1-14* cells were released from a G_1 block at 37°C in the presence of nocodazole, Rad52 focus formation was greatly reduced (Fig. 8G), supporting the hypothesis that premature chromosome segregation can be the cause of DNA break formation in Mec1-deficient cells.

DISCUSSION

Why cells deficient for the S-phase checkpoint die in the presence of replication stress is a long-standing question. Here we show that reducing Cdk1 activity suppresses the loss of viability and the DNA replication defects of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ mutant cells that are treated with low HU doses. Furthermore, reducing the Cdk1 activity bypasses the essential function of Mec1 and Rad53 kinases during an unchallenged S phase. Thus, the execution of some Cdk1-dependent events is detrimental when the S-phase checkpoint is not functional independently of the presence of exogenous replication impediments, suggesting that the essential function of Mec1 and Rad53 is not necessarily separated from the function they exert under replicative stress conditions. Although our work has been carried out in budding yeast, lowering Cdk1 activity appears to counteract the deleterious effects caused by a deficient DNA damage checkpoint also in mammalian cells. In fact, downregulation of the Cdk activator CDC25A rescues the replicative stress occurring after inhibition of the checkpoint proteins Chk1 and ATR (3, 51, 57).

The Cdk1-dependent lethality of *mec1* and *rad53* mutants involves a function of Cdk1 in G_1. In fact, the lack of the G_1 cyclin Cln2, which causes a G_1/S delay by impairing proteolysis of the Clb-Cdk1 specific inhibitor Sic1 (17), suppresses the sensitivity of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells to low HU doses. Accordingly, the deletion of *SIC1*, which abrogates the G_1/S delay caused by the *cln2*Δ mutation (17), counteracts the ability of *cln2*Δ to

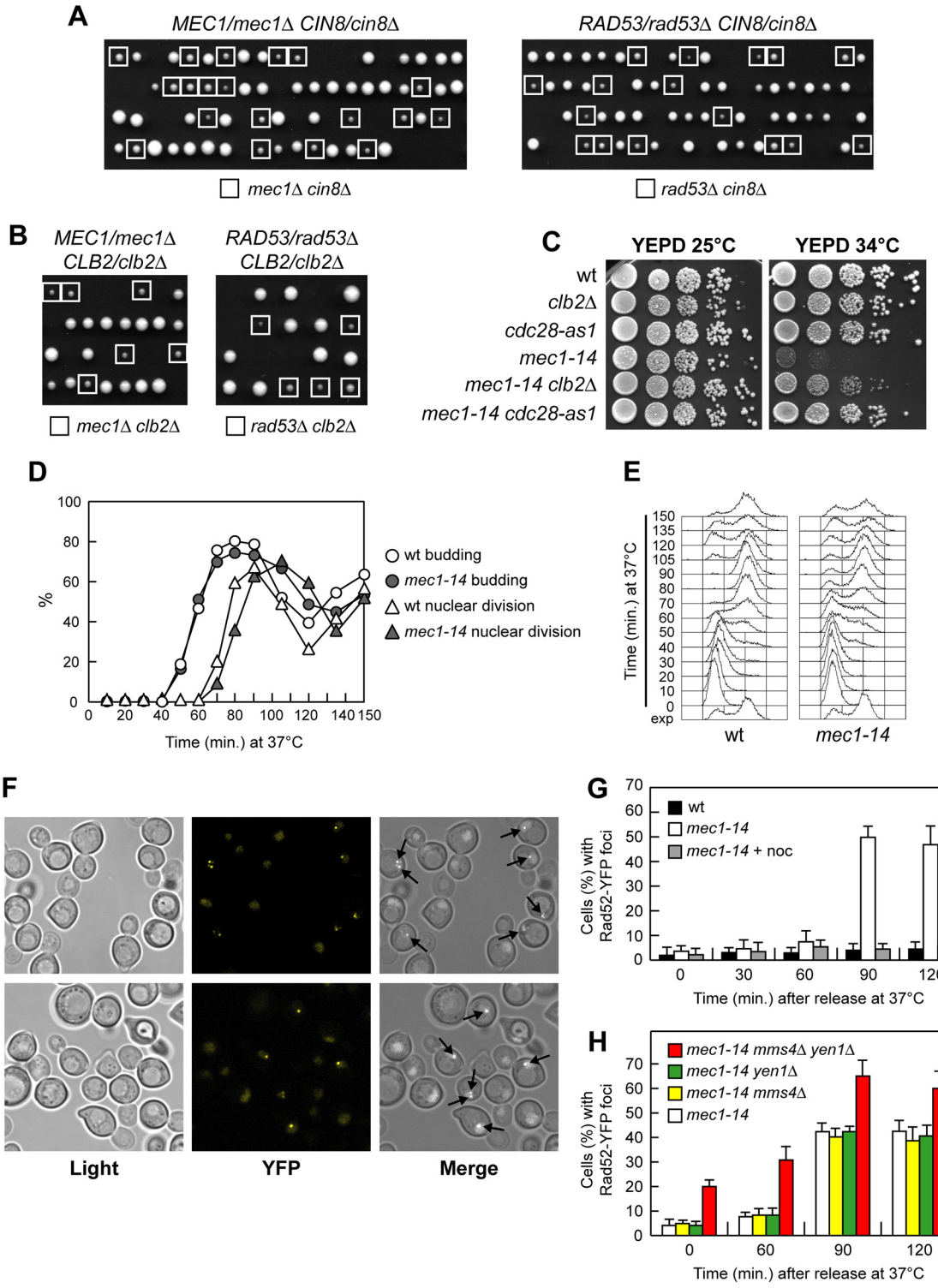

FIG 8 Delaying chromosome segregation bypasses the essential function of Mec1 and Rad53. (A and B) Meiotic tetrads from *MEC1*/*mec1*Δ *CIN8*/*cin8*Δ and *RAD53*/*rad53*Δ *CIN8*/*cin8*Δ diploid strains (A) and from *MEC1*/*mec1*Δ *CLB2*/*clb2*Δ and *RAD53*/*rad53*Δ *CLB2*/*clb2*Δ diploid strains (B) were dissected on YEPD plates, which were then incubated for 3 days at 25°C. Clones from double-mutant spores are highlighted by squares. (C) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates, which were then incubated at the indicated temperatures. (D to G) Cultures of wild-type and *mec1-14* cells expressing a Rad52-YFP fusion were arrested in G_1 with α-factor at 25°C and then released into fresh medium at 37°C. Cell samples were taken at the indicated times after α-factor release for scoring budding and nuclear division (D), for FACS analysis of DNA content (E), and for visualizing Rad52-YFP foci by fluorescence microscopy (F and G). (F) Rad52-YFP foci in *mec1-14* cells after 90 min at 37°C. Arrowheads in the merge mark Rad52 foci relative to cells. (G) Quantitation of Rad52-YFP foci. Half of the *mec1-14* G_1-arrested cell culture was also released into fresh medium at 37°C in the presence of 5 μg of nocodazole/ml (*mec1-14* + noc), and samples were taken to determine the fraction of cells containing Rad52-YFP. (H) G_1-arrested cell cultures were released into fresh medium at 37°C, and Rad52-YFP foci were quantified at the indicated times after α-factor release. The plotted values in panels G and H are mean values ± the standard deviations from three independent experiments. At least 200 cells were counted for each cell culture at each time point.

suppress the HU sensitivity of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells. Thus, a delay in S-phase entry appears to support cell survival to replication stress in the absence of Mec1 or Rad53. This finding is complementary to earlier observations in mammalian systems, where ATR and Chk1 inhibitors were shown to be particularly toxic for p53-deficient cells, which lack the G_1 checkpoint and therefore experience a less restrictive S-phase entry in the presence of DNA damage (42, 57).

It has been shown that Mec1 and Rad53 stimulate production of dNTPs by inducing the expression of the *RNR* genes at the G_1/S transition via inactivation of the transcriptional repressor Crt1 (25). Indeed, the lack of Crt1 not only bypasses the essential function of Mec1 and Rad53 (25) but also improves survival in response to HU treatment of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ mutant cells (Fig. 3D). Therefore, the role of Mec1 and Rad53 in inducing transcription of the *RNR* genes contributes to maintain cell viability also under replication stress. In this scenario, the S-phase delay in *cln2*Δ cells could provide *mec1* and *rad53* mutants with a longer time to reach high enough dNTP levels to support DNA replication. Consistent with previous data showing that increased dNTP pools promote replication of MMS-damaged DNA (9, 40), the lack of Cln2 or Crt1 improves survival of *mec1* mutants also to MMS treatment.

The Cdk1-dependent lethality of *mec1* and *rad53* mutants cannot be entirely ascribed to the G_1 function of Cdk1. In fact, the lack of the mitotic cyclin Clb2 also improves the viability of HU-treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ mutants. Although *mec1* and *rad53* mutants are unable to complete DNA replication in the presence of HU, they prematurely undergo spindle elongation due to upregulation of the kinesin motor protein Cin8 (27). Because Cdk1 activity is required to assemble a mitotic spindle by restraining Cin8 proteolysis (13), a reduced Cdk1 activity might suppress the sensitivity of *mec1* and *rad53* mutants to replication stress by delaying spindle elongation. Indeed, we found that both restraining spindle extension by *CIN8* deletion and disrupting kinetochore-microtubule attachments by nocodazole improve the survival in response to HU of *mec1* and *rad53* mutants. Interestingly, the lack of Cin8 allows *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells to complete DNA synthesis in the presence of HU, suggesting that the DNA replication defects in these mutants can be overcome by providing cells with additional time to complete DNA replication before chromosome segregation takes place. This hypothesis implies that the segregation of incompletely replicated DNA contributes to kill HU-treated *mec1* and *rad53* mutants. Because HU-treated *mec1*Δ *sml1*Δ cells can duplicate their centromeres (19) and therefore are proficient for bipolar attachment that generates tension within the spindle, this precocious spindle elongation can lead to lethal chromosome breaks due to disjunction of incompletely replicated chromosomes (19). Although the force exerted by a bipolar spindle might be insufficient to generate chromosome breakage, the presence of ssDNA in HU-treated *mec1* and *rad53* mutants (20, 50) may cause spindle-induced breakage.

Interestingly, the lack of Cin8 suppresses the sensitivity to HU of *mec1* and *rad53* cells more efficiently than nocodazole addition. Indeed, nocodazole disrupts the kinetochore-microtubule attachment, thus causing the release of the kinetochores from the spindle pole bodies to which they were first attached. It has been shown that premature spindle elongation caused by Cin8 deregulation in nocodazole-treated cells leads to mis-segregation of sister chromatids because it impairs the recapture and biorientation of microtubules after nocodazole removal (10, 31). Thus, one possibility is that the unrestrained Cin8-dependent spindle elongation in HU-treated *mec1* and *rad53* mutants limits the ability of nocodazole to rescue *mec1* and *rad53* cell lethality in HU.

Noteworthy, although *mec1*Δ *sml1*Δ cells are more sensitive to HU and MMS than *rad53*Δ *sml1*Δ cells, both nocodazole addition and *CIN8* deletion improve survival to HU of *mec1* cells more efficiently than that of *rad53* cells. Furthermore, delaying spindle elongation suppresses the MMS sensitivity of *mec1*Δ *sml1*Δ cells, but it only slightly reduces that of *rad53*Δ *sml1*Δ cells. Although HU slows down DNA synthesis by depleting dNTPs, MMS-induced lesions block the progression of DNA replication forks because replicative polymerases cannot accommodate 3-methyl-adenine in their catalytic sites (22, 39). Interestingly, deletion of the *EXO1* nuclease gene has no effect on the sensitivity of *mec1* mutants to MMS, whereas it suppresses cell lethality and the fork progression defects of MMS-treated *rad53*Δ *sml1*Δ cells (48). It is therefore tempting to propose that, while the Mec1 requirement in supporting replication of MMS-damaged DNA can be bypassed by providing additional time to complete DNA replication, the lack of Rad53 results in defects at the replication forks that become substrates for irreversible Exo1-dependent replication fork breakdown or resection events. In line with this view, it has been shown that Exo1 can process aberrant DNA intermediates in *rad53*-deficient cells (12) and that Rad53-dependent phosphorylation of Exo1 may act to limit ssDNA accumulation (36).

In any case, delaying spindle elongation can rescue the HU-induced lethality of *mec1* and *rad53* mutants only during exposure to low HU doses. This finding is consistent with previous data showing that the addition of nocodazole does not improve viability of *rad53* and *mec1* cells following transient exposure to a high HU dose (200 mM) (16, 55). Thus, high HU levels seem to irrecoverably commit checkpoint mutants to death, possibly because they induce the stalling of the replication forks that are defective in restarting replication (15) and/or can undergo irreversible pathological transitions, such as the replisome dissociation from the nascent DNA chains (11, 26, 34).

Is the essential function of the S-phase checkpoint related to its function in supporting DNA replication under mild replication stress? Mec1 and Rad53 are essential for cell viability, and their essential function can be bypassed by increasing dNTP levels through *SML1* deletion (64), suggesting that the lethality of *mec1*- and *rad53*-null mutants is due to DNA replication occurring in the absence of adequate dNTP accumulation. We found that, like the lack of Cln2 (59) (Fig. 3B), the lack of Clb2 or Cin8 not only suppresses the sensitivity of *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells to replication stress but also bypasses the essential function of Mec1 and Rad53. Therefore, cells lacking *MEC1* or *RAD53*, but carrying wild-type *SML1*, appear to die through a mechanism similar to that killing *mec1*Δ and *rad53*Δ cells exposed to low HU doses in the absence of the Sml1-mediated inhibition of RNR activity. In both cases, cells experience defective DNA replication caused by a condition of nucleotide depletion, and cell death can be overcome by providing cells with additional time to accumulate dNTPs and accomplish DNA replication before chromosome segregation takes place. As proposed for HU-treated *mec1*Δ *sml1*Δ and *rad53*Δ *sml1*Δ cells (19), partition of incompletely replicated DNA in *mec1*Δ *SML1* and *rad53*Δ *SML1* cells could give rise to lethal chromosome breaks. Consistent with this hypothesis, conditional inactivation of Mec1 by temperature-sensitive *mec1* al-

leles gives rise to the accumulation of chromosome breakage (8) and of Rad52 foci that can be reversed by nocodazole addition (Fig. 8G).

In summary, we demonstrate that Cdk1-driven events impair cell viability when the S-phase checkpoint is not functional. Given that some cancer cells (such as those expressing oncogenes or lacking tumor suppressors) undergo high levels of replicative stress and that Cdk inhibitors are considered relevant candidates as anticancer drugs, this link between the S-phase checkpoint and Cdk1 activity may be important in developing new targeted strategies improving the efficiency of cancer treatments.

ACKNOWLEDGMENTS

We thank J. Diffley, R. Kolodner, S. Piatti, and R. Rothstein for providing yeast strains. We are grateful to M. Bazzi for preliminary data, L. D'Alfonso and M. Collini for valuable advice at the microscope, M. Lopes and M. Foiani for helpful discussions, and M. Clerici and S. Piatti for helpful suggestions and critical reading of the manuscript.

This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (grant IG11407), Cofinanziamento 2008 MIUR/Università di Milano-Bicocca to M.P.L., and Cofinanziamento 2009 MIUR/Università di Milano-Bicocca to G.L. N.M. was supported by a fellowship from Fondazione Italiana per la Ricerca sul Cancro.

REFERENCES

1. **Allen JB, Zhou Z, Siede W, Friedberg EC, Elledge SJ.** 1994. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8**:2401–2415.
2. **Bastos de Oliveira FM, Harris MR, Brazauskas P, de Bruin RA, Smolka MB.** 2012. Linking DNA replication checkpoint to MBF cell-cycle transcription reveals a distinct class of G_1/S genes. EMBO J. **31**:1798–1810.
3. **Beck H, et al.** 2010. Regulators of cyclin-dependent kinases are crucial for maintaining genome integrity in S phase. J. Cell Biol. **188**:629–638.
4. **Bishop AC, et al.** 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature **407**:395–401.
5. **Branzei D, Foiani M.** 2010. Maintaining genome stability at the replication fork. Nat. Rev. Mol. Cell. Biol. **11**:208–219.
6. **Brown EJ, Baltimore D.** 2000. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev. **14**:397–402.
7. **Canman CE.** 2001. Replication checkpoint: preventing mitotic catastrophe. Curr. Biol. **11**:121–124.
8. **Cha RS, Kleckner N.** 2002. ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science **297**:602–606.
9. **Chabes A, et al.** 2003. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell **112**:391–401.
10. **Chai CC, Teh EM, Yeong FM.** 2010. Unrestrained spindle elongation during recovery from spindle checkpoint activation in *cdc15-2* cells results in mis-segregation of chromosomes. Mol. Biol. Cell **21**:2384–2398.
11. **Cobb JA, Bjergbaek L, Shimada K, Frei C, Gasser SM.** 2003. DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. EMBO J. **22**:4325–4336.
12. **Cotta-Ramusino C, et al.** 2005. Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mol. Cell **17**:153–159.
13. **Crasta K, Huang P, Morgan G, Winey M, Surana U.** 2006. Cdk1 regulates centrosome separation by restraining proteolysis of microtubule-associated proteins. EMBO J. **25**:2551–2563.
14. **de Klein A, et al.** 2000. Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. Curr. Biol. **10**:479–482.
15. **De Piccoli G, et al.** 2012. Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. Mol. Cell **45**:696–704.
16. **Desany BA, Alcasabas AA, Bachant JB, Elledge SJ.** 1998. Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. **12**:2956–2970.
17. **Dirick L, Böhm T, Nasmyth K.** 1995. Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. EMBO J. **14**:4803–4813.
18. **Epstein CB, Cross FR.** 1992. CLB5: a novel B cyclin from budding yeast with a role in S phase. Genes Dev. **6**:1695–1706.
19. **Feng W, Bachant J, Collingwood D, Raghuraman MK, Brewer BJ.** 2009. Centromere replication timing determines different forms of genomic instability in *Saccharomyces cerevisiae* checkpoint mutants during replication stress. Genetics **183**:1249–1260.
20. **Feng W, et al.** 2006. Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. Nat. Cell Biol. **8**:148–155.
21. **Gibson DG, Aparicio JG, Hu F, Aparicio OM.** 2004. Diminished S-phase cyclin-dependent kinase function elicits vital Rad53-dependent checkpoint responses in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **24**:10208–10222.
22. **Green CM, Lehmann AR.** 2005. Translesion synthesis and error-prone polymerases. Adv. Exp. Med. Biol. **570**:199–223.
23. **Halazonetis TD, Gorgoulis VG, Bartek J.** 2008. An oncogene-induced DNA damage model for cancer development. Science **319**:1352–1355.
24. **Hoyt MA, He L, Loo KK, Saunders WS.** 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. J. Cell Biol. **118**:109–120.
25. **Huang M, Zhou Z, Elledge SJ.** 1998. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell **94**:595–605.
26. **Katou Y, et al.** 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature **424**:1078–1083.
27. **Krishnan V, Nirantar S, Crasta K, Cheng AY, Surana U.** 2004. DNA replication checkpoint prevents precocious chromosome segregation by regulating spindle behavior. Mol. Cell **16**:687–700.
28. **Kühne C, Linder P.** 1993. A new pair of B-type cyclins from *Saccharomyces cerevisiae* that function early in the cell cycle. EMBO J. **12**:3437–3447.
29. **Labib K.** 2010. How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev. **24**:1208–1219.
30. **Lisby M, Barlow JH, Burgess RC, Rothstein R.** 2004. Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. Cell **118**:699–713.
31. **Liu H, Liang F, Jin F, Wang Y.** 2008. The coordination of centromere replication, spindle formation, and kinetochore-microtubule interaction in budding yeast. PLoS Genet. **4**:e1000262. doi:10.1371/journal.pgen.1000262.
32. **Longhese MP, Fraschini R, Plevani P, Lucchini G.** 1996. Yeast *pip3*/*mec3* mutants fail to delay entry into S phase and to slow DNA replication in response to DNA damage, and they define a functional link between Mec3 and DNA primase. Mol. Cell. Biol. **16**:3235–3244.
33. **Lopes M, et al.** 2001. The DNA replication checkpoint response stabilizes stalled replication forks. Nature **412**:557–561.
34. **Lucca C, et al.** 2004. Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. Oncogene **23**:1206–1213.
35. **Matos J, Blanco MG, Maslen S, Skehel JM, West SC.** 2011. Regulatory control of the resolution of DNA recombination intermediates during meiosis and mitosis. Cell **147**:158–172.
36. **Morin I, et al.** 2008. Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. EMBO J. **27**:2400–2410.
37. **Nordlund P, Reichard P.** 2006. Ribonucleotide reductases. Annu. Rev. Biochem. **75**:681–706.
38. **Paciotti V, Clerici M, Scotti M, Lucchini G, Longhese MP.** 2001. Characterization of *mec1* kinase-deficient mutants and of new hypomorphic *mec1* alleles impairing subsets of the DNA damage response pathway. Mol. Cell. Biol. **21**:3913–3925.
39. **Paulovich AG, Hartwell LH.** 1995. A checkpoint regulates the rate of progression through S phase in *Saccharomyces cerevisiae* in response to DNA damage. Cell **82**:841–847.
40. **Poli J, et al.** 2012. dNTP pools determine fork progression and origin usage under replication stress. EMBO J. **31**:883–894.
41. **Rahal R, Amon A.** 2008. Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation. Genes Dev. **22**:1534–1548.
42. **Reaper PM, et al.** 2011. Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat. Chem. Biol. **7**:428–430.
43. **Richardson HE, Wittenberg C, Cross F, Reed SI.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59**:1127–1133.
44. **Roof DM, Meluh PB, Rose MD.** 1992. Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. **118**:95–108.

45. **Santocanale C, Diffley JF.** 1998. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature **395**:615–618.
46. **Schwob E, Nasmyth K.** 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. Genes Dev. **7**:1160–1175.
47. **Schwob E, Böhm T, Mendenhall MD, Nasmyth K.** 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G_1-to-S transition in *Saccharomyces cerevisiae*. Cell **79**:233–244.
48. **Segurado M, Diffley JF.** 2008. Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. Genes Dev. **22**:1816–1827.
49. **Shirahige K, et al.** 1998. Regulation of DNA-replication origins during cell cycle progression. Nature **395**:618–621.
50. **Sogo JM, Lopes M, Foiani M.** 2002. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science **297**:599–602.
51. **Sørensen CS, Syljuåsen RG.** 2012. Safeguarding genome integrity: the checkpoint kinases ATR, CHK1, and WEE1 restrain CDK activity during normal DNA replication. Nucleic Acids Res. **40**:477–486.
52. **Straight AF, Sedat JW, Murray AW.** 1998. Time-lapse microscopy reveals unique roles for kinesins during anaphase in budding yeast. J. Cell Biol. **143**:687–694.
53. **Sun Z, Fay DS, Marini F, Foiani M, Stern DF.** 1996. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. **10**:395–406.
54. **Surana U, et al.** 1991. The role of *CDC28* and cyclins during mitosis in the budding yeast *Saccharomyces cerevisiae*. Cell **65**:145–161.
55. **Tercero JA, Diffley JF.** 2001. Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature **412**:553–557.
56. **Tercero JA, Longhese MP, Diffley JF.** 2003. A central role for DNA replication forks in checkpoint activation and response. Mol. Cell **11**:1323–1336.
57. **Toledo LI, et al.** 2011. A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nat. Struct. Mol. Biol. **18**:721–727.
58. **Travesa A, et al.** 2012. DNA replication stress differentially regulates G_1/S genes via Rad53-dependent inactivation of Nrm1. EMBO J. **31**:1811–1822.
59. **Vallen EA, Cross FR.** 1999. Interaction between the *MEC1*-dependent DNA synthesis checkpoint and G1 cyclin function in *Saccharomyces cerevisiae*. Genetics **151**:459–471.
60. **Verma R, et al.** 1997. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. Science **278**:455–460.
61. **Vernis L, Piskur J, Diffley JF.** 2003. Reconstitution of an efficient thymidine salvage pathway in *Saccharomyces cerevisiae*. Nucleic Acids Res. **31**:e120.
62. **Weinert TA, Kiser GL, Hartwell LH.** 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. **8**:652–665.
63. **Zegerman P, Diffley JF.** 2009. DNA replication as a target of the DNA damage checkpoint. DNA Repair **8**:1077–1088.
64. **Zhao X, Muller EG, Rothstein R.** 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell **2**:329–340.
65. **Zhou Z, Elledge SJ.** 1993. DUN1 encodes a protein kinase that controls the DNA damage response in yeast. Cell **75**:1119–1127.