Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway

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RAD53 and MEC1 are essential genes required for the transcriptional and cell cycle responses to DNA damage and DNA replication blocks. We have examined the essential function of these genes and found that their lethality but not their checkpoint defects can be suppressed by increased expression of genes encoding ribonucleotide reductase. Analysis of viable null alleles revealed that Mec1 plays a greater role in response to inhibition of DNA synthesis than Rad53. The loss of survival in mec1 and rad53 null or point mutants in response to transient inhibition of DNA synthesis is not a result of inappropriate anaphase entry but primarily to an inability to complete chromosome replication. We propose that this checkpoint pathway plays an important role in the maintenance of DNA synthetic capabilities when DNA replication is stressed.

[Key Words: DNA replication; S-phase; checkpoint pathway; ribonucleotide reductase; nucleotide levels]

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The fidelity of DNA replication is critical to the proper duplication of a cell. Not only must cells replicate chromosomes, they must do so with great accuracy; without stretches of unreplicated DNA, without gaps, without replicational slippage in repetitive regions, without recombination causing rearrangements, and without breaks. S phase, the period of the cell cycle during which DNA is replicated, is a period of great vulnerability for a cell. Many complicated processes are undertaken during S phase, including the complete unwinding and replication of enormously complex DNA molecules, and chances for cataclysmic error are high. Interference with DNA replication by DNA damage, nucleotide depletion or imbalance, or polymerase malfunction can lead to a number of deleterious events, including increased mutagenesis, chromosome instability, gene amplification, microsatellite instability, and hyper-recombination (Loeb and Kunkel 1982). Each of these events can have severe consequences for an organism, including cell death, birth defects, and cancer. A number of factors cooperate to ensure the fidelity of DNA replication. These include processivity factors, proofreading functions, mismatch repair proteins, a variety of DNA repair activities, and regulatory pathways that sense DNA damage and replicational stress (Loeb and Kunkel 1982). For example, in response to DNA damage and DNA replicational interference, cells induce the transcription of genes that enhance repair capacities and arrest cell cycle progression to provide time for these repair processes to occur (for review, see Elledge 1996). This ensures that DNA replication and segregation—the critical events that allow genetic damage to become irreversibly inherited—are delayed until optimal repair can be achieved. In eukaryotes, these regulatory pathways are called checkpoints.

Checkpoint pathways ensure the proper order and timing of cell cycle events, and compromising these pathways contributes to genomic instability and cancer. The outline of the DNA damage response checkpoint pathway in mammals is emerging. ATM (ataxia telangiectasia mutated), a central player, is a member of the lipid kinase family of proteins and is likely a transducer of a DNA damage signal (for review, see Elledge 1996). ATM controls the timely activation of p53, a transcription factor that activates transcription of the cdk inhibitor p21 (Kastan et al. 1992). Cells defective for any of these genes show a defect in G1 arrest in response to DNA damage, and ATM mutants are also defective in G2 arrest and display radioreistant DNA synthesis. The roles of p53 and ATM in tumorigenesis underscore the importance of the DNA damage response to organismal homeostasis. In the case of ATM, there are additional phenotypes that include specific neural degeneration (Friedberg et al. 1995; Meyn 1995). Recently, an additional mammalian checkpoint gene encoding a protein kinase, Chk1, has been identified (Flaggs et al. 1997; Sanchez et al. 1997). Mammalian Chk1 is phosphorylated in response to DNA damage and is capable of phosphorylating Cdc25C on an inhibitory serine residue (Peng et al. 1997; Sanchez et al. 1997). The fission yeast Chk1 homolog acts downstream of the ATM homolog Rad3 (Walworth et al. 1993; Ford et al. 1994; Carr et al. 1995, Walworth and Bernards 1996; Furnari et al. 1997).
In the budding yeast Saccharomyces cerevisiae a number of genes have been identified that control the ability of cells to arrest the cell cycle and/or activate the transcrip-
tional response. Upstream regulators involved in early steps in this pathway include RAD9, RAD17, RAD24, and MEC3, which are required for cell cycle ar-
rest in G1 and G2 in response to DNA damage. POL2, encoding DNA polymerase 2, DPB11, and RFC5 are up-
stream components of the cell cycle arrest and transcrip-
tion pathways that respond to replication blocks (Elledge 1996). Checkpoint signal transducers include MEC1 and RAD53, which are required for the S-phase checkpoint as well as the transcriptional and G1 and G2 arrest responses to DNA damage (Allen et al. 1994; Kato and Ogawa 1994; Weinert et al. 1994). DUN1, which encodes a protein kinase that is activated in response to DNA damage and replication blocks in a MEC1- and RAD53-dependent manner (Allen et al. 1994), is necessary for the transcriptional response (Zhou and Elledge 1993) and plays a partial role in the G2 arrest in response to DNA damage (Pati et al. 1997). MEC1 belongs to the same subfamily of proteins as ATM, underscoring the evolutionary conservation of this pathway (Greenwell et al. 1995; Morrow et al. 1995). MEC1 and TEL1 regulate the phosphorylation of the Rad53p kinase in response to DNA damage and replication blocks (Sanchez et al. 1996; Sun et al. 1996).

Whereas MEC1 and RAD53 control both the transcriptional and cell cycle responses to DNA damage and replication blocks, it is not clear whether these are the only roles these proteins carry out or whether these proteins play equivalent roles in these responses. In addition, the issue of whether these genes coordinate DNA replication and mitosis in an unperturbed cycle or only in response to replicational stress remains to be resolved. Both genes are essential for viability, perhaps suggesting a role for the checkpoint in each cell cycle, but to date their essential roles have remained obscure. In this study we sought to determine the essential functions of RAD53 and MEC1 by isolation of dosage suppressors of the null allele of rad53. We have discovered that increasing dNTP synthetic capacity can suppress both rad53 and mec1 null alleles. Furthermore, the primary lethal defect in these mutant strains in response to nucleotide depletion is not mitotic entry but a profound defect in the ability to finish chromosomal replication. We propose that one of the roles of this checkpoint pathway is the stabilization of replication structures under conditions of replication inhibition.

**Results**

**RN1 overexpression suppresses Δrad53 and Δmec1 lethality**

To investigate the essential function of the S-phase checkpoint, we selected dosage suppressors of the lethality associated with a deletion of RAD53. A TRP1 2µ S. cerevisiae cDNA library under control of the GAL1 promoter (Mulligan and Elledge 1994) was constructed in λTRP, converted to plasmid form by cre-lox automatic subcloning (Elledge et al. 1991) and used to transform a rad53 null strain, Y324, being kept alive by RAD53 on a URA3 CEN plasmid, pJA92 (Allen et al. 1994). Transformants were selected on synthetic complete medium lacking tryptophan (SC-Trp), with galactose as a carbon source to induce cDNA expression, and replica plated onto the same medium containing 5-fluoro-orotic acid (5-FOA) to select for strains able to grow in the absence of pJA92. We subsequently examined the ability of these 5-FOA' transformants to grow with glucose as the carbon source. Because GAL-driven RAD53 is capable of sustaining cell growth under repressed conditions (glucose), choosing only clones that exhibited partial galactose dependence eliminated both the RAD53 background and any plasmid-independent extragenic suppressors. Twelve clones were at least partially dependent on gal-
actose for suppression of Δrad53. These plasmids were sequenced and the identities of the encoded genes are listed in Table 1, along with the efficiency with which they suppress the growth defect of rad53 deletion mutants. We called those genes SRL, for suppressors of rad53 lethality. A variety of genes are capable of suppressing Δrad53 to varying extents, including a number of transcription factors, both positive and negative. Those suppressors are likely to rescue the lethality indirectly, through effects on the transcription of other genes. Two suppressors are putative 26S proteasome components and are also likely to be indirect suppressors that act by changing the stability of other proteins that suppress the lethality of the rad53 deletion. Other suppressors consist of a protein kinase (MCK1), a putative chaperone (PDR13), and the regulatory subunit of ribonucleoside diphosphate reductase (RNR1). The remainder, designated SRL1, SRL2, and SRL3, show no similarity to other proteins in the database.

RN1 overexpression suppresses mec1, indicating a common essential function for RAD53 and MEC1

Because RAD53 and MEC1 operate in the same checkpoint pathway (Sanchez et al. 1996; Sun et al. 1996), it is possible that they are essential for the same reason. In an effort to determine whether these genes have the same essential function, we examined the SRL genes for their ability to suppress Δmec1 lethality. Most of the suppressors were capable of suppressing the mec1 deletion mutant, albeit poorly. Only one plasmid was able to efficiently suppress both the rad53 and mec1 deletion mutants (Table 1). This plasmid contained the RN1 gene encoding a predicted protein product starting with amino acid 22 of Rnr1 and continuing to the end of the 888-amino-acid protein. RN1 was also shown to suppress the lethality of the Δmec1Δtel1 and Δmec1Δrad53 double mutants (data not shown). RN1 is an essential gene that encodes the large subunit of ribonucleoside diphosphate reductase (RNR), the rate-limiting enzyme of deoxyribonucleotide synthesis and the target of the DNA synthesis inhibitor hydroxyurea (HU). RN1 is both inducible by DNA damage and tightly cell cycle
regulated (Elledge and Davis 1990). A gene encoding an alternative large subunit of Rnr, RNR3, is a target gene of the DNA damage and replication interference response pathways and is 80% identical to RNR1 at the amino acid level. We found that full-length RNR1 and RNR3 are both able to efficiently suppress Δrad53 and Δmec1 when expressed from the constitutive GAP promoter on a 2μ plasmid (pGAP–RNR1, pGAP–RNR3; Fig. 1A).

Low levels of ectopic RNR1 can suppress lethality
To examine whether up-regulation of RNR1 or RNR3 was the mechanism through which the other suppressors functioned, Northern analysis was performed on total RNA isolated from asynchronously growing cultures of each suppressed Δrad53 strain. There were no large increases in either RNR1 or RNR3 mRNA levels between wild-type cells and the suppressors (Fig. 1B–D), with the exception of Ssn6. There is a three-fold increase in RNR3 expression when truncated Ssn6 protein is expressed. RNR3 is negatively regulated by Ssn6 (Zhou and Elledge 1992); therefore, this truncated Ssn6 might be acting as a dominant-negative mutant. The more general failure to detect strong differences in RNR transcription does not completely rule out altered RNR expression as a mechanism of suppression because very low amounts of exogenously supplied Rnr1 are still capable of suppressing Δrad53. For example, RNR1 under GAL1 control can still suppress when grown on glucose (data not shown). Additional support comes from the fact that one additional copy of the RNR1 gene under its own promoter is capable of efficient suppression, indicating that a twofold increase in RNR1 gene dosage is sufficient for suppression.

Mec1 has a greater role than Rad53 in response to genotoxic stress
Mec1 and Rad53 are both required for the transcriptional and cell cycle arrest responses to DNA damage and replication blocks. However, it was unclear whether they were equivalent in these functions because only hypomorphic alleles could be compared because of their essential nature. Having common suppressors of mec1 and rad53 null mutations allowed us to examine the phenotypes associated with a complete loss of function. In addition to defects in cell cycle arrest and transcriptional responses, previously isolated point mutants of RAD53 and MEC1 show a high degree of sensitivity to UV and ionizing radiation, radiomimetic drugs, and HU. Δrad53 +pGAP–RNR1 cells show the same degree of sensitivity to HU and UV irradiation as rad53-21 point mutants (Fig. 2A,B, circles). In addition, analysis of spindle elongation in α-factor-synchronized rad53-21 and rad53 cells released into media containing HU indicated that both of these alleles confer equivalent defects in the S-phase checkpoint (Fig. 2C,D, circles). The rad53 null mutant actually exhibits a slower rate of accumulation of anaphase-like spindles, but this parallels the slower rate of budding that is also observed under these conditions (Fig. 2C, circles). Thus, although RNR1 suppresses the lethality of Δrad53, it is unable to suppress the checkpoint and DNA damage sensitivity associated with loss of Rad53 function. This suggests that RNR1 overexpression allows rad53 (and mec1) null cells to tolerate an altered cellular physiology, rather than restoring function to the MEC1 RAD53 pathway.

Δmec1 +pGAP–RNR1 cells are also defective in the response to DNA damage and replication blocks but more so than the mec1-21 point mutant, suggesting that mec1-21 is still partially competent in some of its responses. When the mec1 and rad53 null strains are compared, it is clear that the Δmec1 mutant is significantly more UV- and HU-sensitive (Fig. 2A,B). This indicates that MEC1 has a greater role in response to DNA damage than does RAD53, which is consistent with the fact that Rad53 is downstream of Mec1 in the pathway and indi-

### Table 1. Summary of rad53 and mec1 deletion suppressors

| ORF name   | Gene name             | Function                  | Strength of suppression of rad53 | ORF size (nt) | Portion cloned (nt) |
|------------|-----------------------|---------------------------|----------------------------------|--------------|---------------------|
| YBR112c    | SSN6/CYC8/CRT8        | transcriptional repressor | weak                             | 2898         | 1-600               |
| YDR173c    | ARGR3/ARG82           | transcriptional repressor/activator | good                           | 1065         | entire              |
| YER070w    | RNR1/CRT7             | ribonucleoside diphosphate reductase | strong                        | 2664         | 66-end              |
| YHR064c    | PDR13                 | drug resistance, Hsp70 family | weak                            | 1716         | 82-end              |
| YJL110c    | GZF3/NIL2             | transcriptional repressor | weak                             | 1653         | 637-end             |
| YKR091w    | SRL3                  | weak                       | 456                              |              | entire              |
| YLR082c    | SRL2                  | good                       | 1176                             |              | entire              |
| YNL307c    | MCK1                  | meiotic protein kinase      | poor                             | 1125         | entire              |
| YOR247w    | SRL1                  | weak                       | 630                              |              | entire              |
| YOR259c    | RPT4/SUG2             | SPB duplication, 26S proteasome | good                         | 1311         | 35-end              |
| YOR261c    | RPN8                  | 26S proteasome             | good                             | 1014         | 76-880              |
| YPL129w    | ANC1/TFG3             | transcription factor        | poor                             | 731          | 161-end             |

Data are from deletions suppressed after 5-FOA selection (Elledge and Davis 1990). Knockouts in MEC1 or RAD53 permitted growth on 5-FOA. A gene encoding an alternative large subunit of Rnr, RNR3, is a target gene of the DNA damage and replication interference response pathways and is 80% identical to RNR1 at the amino acid level. The extent of each ORF that was contained on each library plasmid was approximated using the sequence of the 5′ end of the insert, which was approximated by gel electrophoresis. (nt) Nucleotide.
The amount of RNR1 overproduction does not enhance the rate of DNA replication. Because low levels of additional RNR1 expression are...
capable of suppressing the lethality of mec1 and rad53 mutants, we entertained two general hypotheses for how this suppression might work. The first is based on the assumption that because MEC1 and RAD53 coordinate S-phase completion and mitosis under certain circumstances, their loss may allow S phase and mitosis to occur based on their natural timing, akin to a race between S phase completion and mitotic onset. Thus, by adding additional nucleotides S phase may be shortened to the point where it is completed prior to a lethal mitosis. The second hypothesis is that the MEC1/RAD53 pathway provides a function other than cell cycle coordination, such that the loss of Rad53 and Mec1 creates a special nucleotide stress or a greater sensitivity to normal nucleotide levels—levels that may be suboptimal for DNA polymerization or fork stability. Because rad53 mutants are sensitive to low nucleotide levels, we know that nucleotide depletion is toxic. Although the HU sensitivity is generally assumed to be due to inappropriate mitotic entry, this has not been rigorously demonstrated and other explanations exist. For example, nucleotide depletion sensitivity could result from the occasional disassembly of a paused replication complex searching for nucleotides, and MEC1/RAD53 might help to restore the function of these (transiently) nucleotide-starved complexes. Providing additional nucleotides in the form of RNR overexpression might prevent this stress from occurring. In both hypotheses, RNR1 overexpression suppresses by providing extra dNTPs; in the first case, the dNTPs would suppress by accelerating the rate of S-phase completion, whereas in the second case they would suppress by preventing a cataclysmic response to perceived nucleotide depletion by reversing that depletion.

To test the first hypothesis, we examined whether S phase was shorter in wild-type cells overproducing RNR1 under GAP control. Cells were arrested in G1 with α-factor, released from the block, and their DNA content was measured by FACS analysis at 2 min intervals. Although a very small effect cannot be ruled out, the overexpression of RNR1 had no apparent effect on the timing of S-phase completion or the overall rate of DNA synthesis (Fig. 4).
Delaying mitosis cannot rescue the lethality of mec1 and rad53 null mutants

If the outcome of a race between S phase and mitosis determines lethality, the result could be influenced not only by making S phase happen faster but also by delaying mitosis. To test this we examined the effects of agents capable of delaying mitosis on mec1 and rad53 null mutants.
We germinated spores from a Δrad53::HIS3/RAD53 heterozygous diploid on media containing sublethal amounts of benomyl (15 µg/ml), which delays mitosis through activation of the mitotic spindle assembly checkpoint (Elledge 1996). No His+ colonies were viable under these conditions. We also streaked rad53 null cells containing RAD53 on a URA3 CEN plasmid (pJA92) onto media containing 5-FOA and 15 µg/ml benomyl but observed no increase in the appearance of 5-FOA resistant colonies relative to the absence of benomyl.

mec1 and rad53 null mutants are extremely sensitive to low HU levels on plates. We identified the minimal concentration of HU that blocked growth on plates (5 mM) and attempted, unsuccessfully, to suppress the lethality of either mutant with 15 µg/ml benomyl.

Because survival was measured as growth on plates in previous experiments, the concentration of microtubule inhibitors employed was necessarily not sufficient to completely block mitosis, and low levels of suppression might be obscured as a result. To examine this more thoroughly, we tested the ability of a sustained mitotic block to allow rad53 mutants to recover from a transient HU block (Fig. 5A). rad53-21 mutants were released from a G1 block into media containing 0.25 M HU. After 30 min, the HU was washed out and the cells were resuspended in media containing 80 µg/ml benomyl with no HU, and viability was measured over time. Blocking mitosis with benomyl was unable to restore any measure of viability. The inability of benomyl to rescue either the lethality of the null, or the sensitivity of either the null or the point mutant to HU, suggests that the lethal event may be the same in each case (the consequence of nucleotide depletion) and unrelated to whether or not cells are allowed to proceed into mitosis. This is consistent with the fact that in rad53 and mec1 null mutants, loss of viability in HU does not correlate with the degree of spindle elongation (Fig. 2A,D).

As the cause of lethality in null mutants and HU-treated null and point mutants does not appear to be solely due to the relative timing of S phase and mitosis, it is likely that in rad53 and mec1 null cells a lethal event is occurring late in S phase or early in G2.

Figure 4. RNR1 overproduction does not accelerate progression through S phase. DNA replication timing of Y300 (wild type, trp1-1) and Y580 (TRP1::GAP–RNR1) strains is shown. Cells were grown to log phase at 30°C and arrested with 10 µg/ml a-factor for 3 hr. Upon release from the a-factor block into YPD, samples were taken at close intervals and stained with propidium iodide and analyzed by flow cytometry for the purpose of detecting subtle differences in the rate of replication due to RNR1 overproduction.

Figure 5. Inability of a microtubule inhibitor to suppress the lethality of rad53 mutants transiently exposed to HU. (A) Sensitivity of rad53-21 to HU in the presence of benomyl. A rad53-21 strain, Y301, was released from a-factor arrest into 0.25 M HU for 30 min. Following this transient incubation the culture was maintained in 80 µg/ml benomyl, and timed aliquots were plated onto YPD for measurement of viable colony-forming units. (B) FACS analysis of Y301 (rad53-21) and Y300 (wild-type) cultures that had been transiently treated with HU. Wild-type and rad53-21 cultures were released from the G1 block into either 0.25 M HU for 30 min or medium lacking HU, as indicated. At 30 min after a-factor release, cells were washed and transferred into YPD containing 80 µg/ml benomyl. Progress through S phase was monitored by FACS at the indicated time points.
ring that commits the cells to death regardless of the timing of the subsequent mitosis. As we described earlier, one such event could be defective DNA replication caused by a condition of nucleotide depletion. To determine whether mutant cells transiently arrested with HU did in fact have difficulty finishing DNA replication after removal of the replication block, we examined DNA content in rad53-21 and wild-type cells under these conditions. Although the rad53 mutant showed a delay in replicating its DNA relative to wild-type cells transiently treated with HU, it eventually accumulated with an approximately G2 DNA content (Fig. 5B), indicating that it recovered the ability to produce sufficient dNTP levels to replicate a genome's worth of DNA.

Because FACS analysis cannot determine to what extent mitochondrial DNA contributes to the amount of G2 DNA observed in this experiment, we performed a similar experiment in \( r^0 \) rad53-21 mutant strains (Fig. 6A). \( r^0 \) rad53-21 mutants were released from \( a \)-factor into 0.2 M HU and 10 µg/ml nocodazole, the HU was washed away after 1 hr, and samples were analyzed for DNA content for up to 3 hr (Fig. 6B, bottom). Under transient HU-treatment conditions that resulted in 75% lethality (Fig. 6A), we observed the same accumulation of apparent G2 DNA content as in the \( r^+ \) strains (cf. Figs. 6B and 5B). The control experiment in the absence of HU (Figs. 5B and 6B, top) indicates that the effect is specific to HU. The observed delay in replication in rad53-21 mutants was not unexpected because rad53 mutants are unable to induce expression of the RNR1, RNR2, RNR3, and RNR4 genes to quickly increase nucleotide biosynthetic capacity (Allen et al. 1994; Huang and Elledge 1997). Alternatively, the delay could be due to the presence of lesions that occur in the transiently nucleotide-starved cells (e.g., stalled replication complexes or abandoned replication forks) that persist and impede the function of the active replication complexes that subsequently encounter them. These data confirm that rad53-

**Figure 6.** Inability of \( r^0 \) rad53-21 mutants to complete chromosomal replication after a transient HU treatment. Y623 (wild-type \( r^0 \)) and Y624 (rad53-21 \( r^0 \)) cells were arrested in \( a \)-factor for 3 hr and washed into YPD media containing either 10 µg/ml nocodazole or 0.2 M HU and nocodazole. After a 60-min incubation, cells were washed and resuspended into YPD medium containing 10 µg/ml nocodazole only and monitored for viability (A), DNA content (B), and chromosome integrity (C,D). (A) Sensitivity of rad53-21 (●) to transient HU treatment in the presence of nocodazole. Wild-type (●) is shown for comparison. (B) Flow cytometric analysis of the DNA content of wild-type and rad53-21 strains. Transiently HU-treated cultures are shown at bottom, with the asterisk (*) indicating the time at which the cells were washed out of HU. (Top) Cultures released from \( a \)-factor into nocodazole only. (C) CHEF gel of chromosomes from wild-type (left) and rad53-21 (right) strains transiently treated with HU. The vertical bar over each lane indicates time points at which HU was present (shaded bars) or had been washed out (open bars). The two chromosomes that were used in part (D) are indicated. (D) Quantitation of replication of chromosomes from wild-type \( r^0 \) Chr A (●) and \( r^0 \) Chr B (○) and rad53-21 (rad53-21 \( r^0 \) Chr A (●) and rad53-21 \( r^0 \) Chr B (○)) cultures that had been transiently treated with HU and resolved by CHEF in C. The two chromosomes examined are indicated in C. The amount of fully duplicated chromosomes in the rad53 mutants precisely correlates with the percentage survival. Intensities of the bands were quantitated using NIH Image software and plotted as a function of time after release from \( a \)-factor and plotted in arbitrary units.
21 cells are delayed but not deficient in restoring DNA synthetic capability after transient HU treatment. However, the cells are clearly dying, and forestalling mitosis with microtubule destabilizing drugs has no effect on this.

FACS analysis measures only bulk DNA content, and it cannot determine whether a small percentage of the DNA is unreplicated or, in the case of the previous experiment, whether the apparently replicated chromosomes at the end of the experiment are intact. To examine the integrity of chromosome structure, we employed pulsed-field gel electrophoretic (PFGE) analysis. Incompletely replicated chromosomes fail to enter a pulsed-field gel because of the presence of forks and replication bubbles that impede migration (Hennessy et al. 1991). Chromosomal DNA was prepared from the cultures of wild-type $p^0$ and rad53-21 $p^0$ mutant cells that had been treated transiently with HU and kept in the presence of nocodazole. At timed intervals, DNA from these cells was prepared and examined by PFGE (Fig. 6C) and quantitated densitometrically (Fig. 6D) (see Materials and Methods). Transient HU treatment delayed the re-entry of chromosomes from wild-type cells, consistent with the kinetics observed by FACS analysis. In contrast, chromosomes from the rad53 mutant never re-entered the gel, even during a 6-hr mitotic block. Similar results were obtained with mec1 mutants (data not shown). Quantitation of the intensities of two chromosome bands, designated A and B, shows that wild-type chromosomes double in intensity from 150 min, indicating completed replication. rad53 chromosomes reappear at 180 min at half the original intensity, indicating that a quarter of the population has properly completed DNA synthesis, consistent with the survival data. This indicates that in addition to experiencing a significant delay in the recovery of bulk DNA synthetic capacity, when the rad53 mutant's chromosomes do eventually become apparently fully replicated (by FACS analysis), they have a profoundly abnormal structure (by PFGE).

Genetic interactions between the checkpoint and origin initiation machinery

We have described defective DNA replication as a consequence of transient nucleotide depletion in checkpoint mutants. Because checkpoint null mutants can be suppressed by increasing nucleotide biosynthetic capacity, it is likely that the null mutants experience a nucleotide depletion and die for the same reason as hypomorphic mutants that experience a transient nucleotide depletion. Therefore, an important issue is the nature of the perceived nucleotide depletion in checkpoint null cells. These mutants could be sensitive to the normal dNTP levels present in each cell cycle, or alternatively, the absence of the checkpoint could create a nucleotide depletion to which the cells cannot subsequently respond. In the latter case, the mechanism could be a direct failure to up-regulate RNR activity or an indirect consequence of a failure to properly regulate the nucleotide consumption of other cellular machinery. While investigating the genetic interactions between checkpoint mutants and origin-firing mutants, we have uncovered support for the idea that timing of origin firing may contribute to the nucleotide depletion that kills checkpoint null mutants. The temperature-sensitive origin firing mutant orc2-1 (Liang et al. 1995) displays an extended duration of S phase upon release from an $\alpha$-factor arrest, even at the permissive temperature (data not shown). To determine whether this might be mimicking the effect that HU has on S phase, we constructed orc2-1 mec1-21 and orc2-1 rad53-21 double mutants. Surprisingly, both double mutants are viable, suggesting that the lengthened S phase in orc2-1 is a qualitatively different phenomenon than that caused by HU treatment, which kills these checkpoint mutants. Even more startling is the fact that the mec1-21 mutation, but not the rad53-21 mutation, can suppress the temperature sensitivity of orc2-1 (Fig. 7A) at 30°C. This observation suggests that the checkpoint pathway is acting antagonistically to the origin-firing defect of orc2-1.

The suppression of orc2 by a mec1 mutation bears on the essential function of the DNA replication checkpoint because if there is an antagonistic interplay between checkpoint genes and origin-firing genes at the level of origin firing, then it could be that inappropriate origin firing in checkpoint null mutants creates a nucleotide depletion that commits the cells to lethality. If true, then origin firing mutants might be expected to abrogate this effect and suppress the lethality of check-

![Figure 7](genesdev.cshlp.org)
point null mutants. The concept that the checkpoint and the origin-firing machinery specifically interact with each other is further supported by recent work (Santocana-le and Diffley 1998, and pers. comm.) indicating that the timing of origin firing is negatively regulated by the DNA replication checkpoint pathway. To explore this idea we examined interactions between the checkpoint pathway and the Dbf4/Cdc7 complex, a protein kinase that is required for origin initiation (Jackson et al. 1993). We tested dbf4-1 and cdc7-1 mutants for suppression of Δrad53 and Δmec1 by isolating double mutants that contained the wild-type alleles of RAD53 or MEC1 on a URA3 plasmid. These strains were struck onto plates containing 5-FOA to assess their ability to grow in the absence of checkpoint gene product. We found that Δmec1 but not Δrad53 was suppressible by dbf4-1 and cdc7-1 (Fig. 7B, data not shown), supporting the plausibility of this idea. Why mec1 and not rad53 mutants would exhibit these interactions with origin firing mutants is not clear, but the explanation may lie in the additional functions of Mec1 somehow impinging on these events or in a more complex relationship between origin firing and checkpoint function, as detailed in the Discussion.

**Discussion**

Cell cycle checkpoints have been thought of primarily as surveillance mechanisms that respond to aberrations in cellular structures, such as DNA damage or replication blocks, and prevent catastrophic cell cycle transitions. Unlike the checkpoint genes specific for DNA damage, those involved in the DNA replication checkpoint are essential for viability. The fact that all known replication interference checkpoint genes in S. cerevisiae are essential is an indication either that events occurring during the course of a normal cell cycle require the coordinating activities of this checkpoint or that the DNA replication checkpoint genes have activities in addition to the cell cycle coordination traditionally thought to be their primary function (Weinert and Hartwell 1988). We investigated this poorly understood aspect of checkpoint function by performing a high copy suppressor screen of the lethal rad53 null mutation. We found that overproduction of RNR1 eliminated the requirement for both MEC1 and RAD53, indicating an interaction between nucleotide levels and checkpoint function even in the absence of nucleotide-depleting drugs. We also determined that lethality caused by nucleotide stress in checkpoint-deficient cells can be attributed to failure of replication structures to completely recover from the immediate effects of nucleotide depletion, suggesting that replicational stress due to suboptimal nucleotide levels may occur during a normal cell cycle.

**Functional distinction between MEC1 and RAD53**

The mec1 and rad53 alleles that were previously available for study were necessarily hypomorphic and not complete loss-of-function alleles. This has made determination of the relative roles played by each in the checkpoint pathway impossible to definitively establish. The existence of a common suppressor allows a direct comparison of the two null mutants with existing hypomorphic alleles and with each other. The UV and HU sensitivities of the mec1-21 mutant are much less severe than the mec1 null mutant, indicating that the mec1-21 allele retains significant residual function. The UV and HU sensitivities of the rad53-21 and rad53 null mutants are very similar. Furthermore, the kinetics and extent of spindle elongation in HU-treated rad53-21 mutants are essentially indistinguishable from that of both rad53 and mec1 null mutants after general cell cycle perturbations are taken into account, indicating that rad53-21 can be considered to be nearly completely defective for the cell cycle delay function.

The major point of similarity between the mec1 and rad53 null strains is the fact that even moderate RNR1 overproduction can efficiently suppress them both. Furthermore, a rad53 mec1 double null mutant is also easily suppressible by RNR1 (data not shown). This indicates that the essential functions of both genes are the same. Moreover, using the common suppressor approach we can state unequivocally that there is a functional distinction between RAD53 and MEC1 observable at the level of sensitivity to UV irradiation and HU treatment, with MEC1 contributing more to resistance than RAD53. Given that the kinetics of anaphase entry of rad53 and mec1 null mutants in the presence of HU are very similar to each other, we believe that the actual cell cycle regulatory functions of the two gene products are therefore also similar but that MEC1 has additional roles required for recovery from replicational stress. This is also consistent with the fact that MEC1 acts upstream of RAD53 in the checkpoint pathway and is required for its phosphorylation in response to DNA damage and replication blocks.

What is the essential function of the S-phase checkpoint?

Whereas RAD53 and MEC1 are essential genes in S. cerevisiae, their homologs in Schizosaccharomyces pombe, cds1" and rad3", respectively, are not (Al-Khodairy et al. 1994; Murakami and Okayama 1995). The MEC1-related gene ATM is also dispensable for cell growth in humans and mice (Barlow et al. 1996; Elson et al. 1996; Xu et al. 1996). This suggests that the essential natures of MEC1 and RAD53 are reflections of a checkpoint requirement that manifests in every cell cycle in S. cerevisiae. Our findings that RNR1 and RNR3, the rate-limiting regulatory subunits of ribonucleotide reductase, are dosage suppressors of the lethality of the mec1 and rad53 null mutations support this idea and indicate that the essential function of these genes involves maintaining an adequate nucleotide supply, as opposed to responding to some kind of DNA damage. The fact that low amounts of exogenously supplied RNR1 can efficiently suppress lethality suggests that the defect responsible for lethality is just below the threshold for survival. However, RNR1
can do little to overcome the effects of exposure to the RN inhibitor HU, which requires full activation of the checkpoint for a prolonged period of time.

dNTPs levels are highly regulated (for review, see Elledge et al. 1992). The mRNA for RNR1 is tightly cell cycle regulated, the mRNAs for all four RNR genes are inducible in response to DNA damage and replication blocks, the substrate specificity of the reductase is modulated by particular dNTPs to ensure an equal supply of all four dNTPs, and dATP feedback inhibits the overall activity of the enzyme to prevent excessive build up of dNTPs. An important question is why the levels of dNTPs in mec1 and rad53 mutants are insufficient for survival. One possibility is that mec1 and rad53 cells are simply more sensitive to normal levels of nucleotides. Perhaps nucleotide levels are normally maintained at a level that is limiting for polymerase function. In vitro it has been shown that high nucleotide levels lead to increased misincorporation rates because proofreading mechanisms have less time to function before the next nucleotide is inserted (Fersht 1979). Thus, it is possible that normal in vivo nucleotide levels cause polymerase pausing in a state that is deleterious in the absence of the replication stress response pathway. A second possibility is that the checkpoint has a direct role in up-regulating dNTP synthesis during S phase such that the loss of checkpoint function would actually cause a nucleotide depletion to which it then would not be able to respond. RAD53 does regulate the transcription of RNR1, RNR2, RNR3, and RNR4 in response to HU treatment and DNA damage; however, the viable rad53-21 allele is completely defective for this transcriptional regulation (Allen et al. 1994; Huang and Elledge 1997), suggesting that this function is not specifically lacking in null mutants. If up-regulation of nucleotide synthesis is regulated by the checkpoint, the defect is not at the level of RNR1 accumulation because RNR1 levels appear to be normal in the null mutants. Furthermore, overproduction of RNR2 and RNR4 fail to suppress rad53 lethality (data not shown). A third possibility is that in the absence of the checkpoint, a secondary event causes a more rapid consumption of dNTPs such that their levels are lower than normal, mimicking HU treatment. This, together with an inability to respond to such a nucleotide depletion, however transient, could cause lethality.

Currently we cannot distinguish between the three models presented in the preceding paragraph. However, the third model, indirect nucleotide depletion as a secondary effect of checkpoint deficiency, has recently gained support. The firing of late replication origins is advanced in rad53 and mec1 mutants (Sanctonale and Difley 1998, and pers. comm.). Consistent with this observation, we found that the mec1-21 point mutant suppresses the temperature sensitivity of mutations in ORC2, a gene required for origin recognition and firing. Normally at the G1-S transition, up-regulation of ribonucleotide reductase and the triggering of replication origins occur by separate but parallel regulatory networks. Yet the activation of replication complexes and the dNTP supply must be coordinated because firing of origins with insufficient nucleotide levels would cause a condition of effective nucleotide deprivation. The S-phase checkpoint pathway may provide this coordination. Failure to do so would result in premature or excessive origin firing as observed in mec1 and rad53 mutants. The presence of more origins replicating DNA at the same time might consume nucleotides faster than they can be synthesized, leading to DNA replicative stress, a checkpoint requiring situation. RNR1 overexpression could alleviate this problem without restoring checkpoint function. We tested this by artificially slowing down origin firing in checkpoint mutant backgrounds using temperature-sensitive dbf4-1, cdc7-1, and orc2-1 mutants. Although these mutants were unable to suppress the lethality of rad53 null mutants, we have found that mutations in dbf4 and cdc7 can suppress the mec1 null mutant. The inability to suppress the rad53 null mutation might indicate a novel role for RAD53 relative to MEC1, or a possible redundancy in RAD53 regulation. We have shown previously that TEL1, a MEC1 homolog, can activate Rad53 to a limited degree (Sanchez et al. 1996). Thus, it is possible that a rad53 null mutant could have a more severe defect than a mec1 null mutant under certain circumstances. In addition, it is possible that dbf4 mutants can suppress the lethality of rad53 null mutations but that the double mutant then dies because of a condition unique to the rad53 null mutation. In support of such a possibility we have observed that dbf4-1 rad53-21 and cdc7-1 rad53-21 double mutants are inviable (B.A. Desany and S.J. Elledge, unpubl.).

The genetic interactions between the checkpoint and origin initiation pathways support the notion that the MEC1/RAD53 pathway is acting antagonistically to the origin firing machinery for the purpose of maintaining coordination between the initiation of DNA replication and the nucleotide supply. Furthermore, we believe that the simplest interpretation of our data is that in the absence of the checkpoint pathway, nucleotide levels become limiting either by increased consumption due to increased origin-firing or by an unknown mechanism, and this situation, together with the ability to properly respond to nucleotide depletion, results in lethality.

What is responsible for lethality in the presence of HU?

Replication checkpoint-defective cells die rapidly when exposed to HU, and inappropriate spindle elongation has been thought to be responsible for this lethality. However, microtubule-inhibiting drugs are incapable of rescuing either the lethality of the mec1 and rad53 null mutants or the HU sensitivity of the point mutants. Additionally, the spindle elongation defects of the mec1 and rad53 null mutants are similar to each other, whereas their sensitivities to HU are significantly different. We interpret this to indicate that spindle elongation, rather than being the sole lethal event in these cells, is being misregulated independently of another event that is irreversibly committing cells to death. This is similar
to the results obtained in S. pombe with mutations in cds1, the gene related most closely toRAD53. cds1 mutants die in response to HU treatment but do not appear to enter mitosis prematurely (Murakami and Okayama 1995; Lindsay et al. 1998). Similar results were obtained with hus1 mutants (Enoch et al. 1992). Although there was no attempt to artificially delay mitotic entry to rescue the lethality in those experiments, it is likely that these mutants are dying for the same reasons as rad53 mutants in HU. Our experiments show that rad53 mutant cells have a reduced ability to synthesize intact chromosomes following transient nucleotide depletion. This is not due to an inability to resume dNTP production because bulk DNA synthesis resumes after the block is removed, albeit with slower kinetics than wild type. Whether the structures that prevent chromosome migration in pulsed field gels are normal replicational intermediates that persist much longer than usual, such as replication forks, or are structurally aberrant in some way because of errors resulting from stalled polymerases is not clear. Stalled replication complexes could occasionally disintegrate and require checkpoint-mediated restoration. Alternatively, the collapse of complexes on converging forks could leave lethal gaps of unreplicated DNA. Aberrant DNA repair could also lead to defective chromosomal structure. Although it is not known whether the MEC1/RAD53 pathway directly controls repair processes, it is clear that HU causes damage because rad51 and rad52 mutants are very sensitive to HU (Allen et al. 1994).

Taken together, our results suggest that viability of rad53 and mec1 null mutations is not due to premature mitotic entry but to an inability to survive with the existing nucleotide levels present in those mutants. Furthermore, our results indicate that the lethality resulting from limiting nucleotides is not purely a cell cycle transition phenomenon but is due instead to the profound inability of these mutants to properly carry out chromosomal replication after transient nucleotide depletion. Although this defect could be caused by misregulation of an as yet unappreciated aspect of cell cycle coordination distinct from anaphase commitment, it is clearly not the onset of anaphase that is causing lethality in these mutants because preventing anaphase cannot restore viability after a transient replication block. We favor the model that the checkpoint pathway is more than a cell cycle response. The fact that mec1 and rad53 null mutants appear to be equally checkpoint defective but have significantly different sensitivities to DNA-damage and replication-blocking agents suggests that this pathway controls repair activities in addition to coordination of cell cycle transitions. In this light, these pathways should be considered to be DNA-damage and DNA replication-block stress-response pathways as opposed to solely concerning themselves with cell cycle transitions.

Materials and methods

Yeast growth conditions

Yeast cells were grown at 30°C unless indicated otherwise. Rich and SC medium was formulated according to Kaiser et al. (1994). The carbon source was glucose, unless indicated, in which case the glucose was replaced by galactose. Where indicated, 5-FOA was used at 0.1%, and benomyl in solid media was used at 15 µg/ml.

Isolation of SRL genes

Strain Y324 (see text and Table 2) was grown in YPD and transformed with a 2µ TRP1 S. cerevisiae cDNA library (ATTC nos. 87288 and 47059) using the lithium acetate method. Transformants were plated on SC – Trp GAL (containing galactose) and replica-plated to SC – Trp GAL supplemented with 5-FOA. Positive clones were tested for their ability to grow on SC – Trp supplemented with 100 mM HU. Negatives were then struck to either YPD or YPD with the glucose replaced by galactose (YP-Gal). Clones that displayed any degree of galactose-dependent growth were tested for repeatability by plasmid rescue and retransformation of Y324, followed by verification of 5-FOA resistance. These final positive clones were christened SRL genes.

RNA purification and Northern blotting

RNA purification and Northern blotting were performed as described (Navas et al. 1995). For detection of the endogenous RNR1 transcript in the presence of exogenously provided RNR1, we used a HindIII–SpeI fragment as a probe corresponding to nucleotides 2642–3317 of the 3’ end of the RNR1 transcript. These sequences are not present on the exogenous RNR1 expression constructs. For detection of CLN2 mRNA, we used a StyI fragment of CLN2 comprising nucleotides 460–1541 of the 1638 nucleotide ORF.

Quantitation of bands was performed by exposing the blots to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) and using ImageQuant software to quantitate the band intensities. In all cases, the lane background was subtracted from each band prior to normalization to the loading control (ACT1).

HU- and UV-killing assays

For HU killing, cultures were grown to log phase in YPD, whereupon the medium was replaced with YPD +0.2 mM HU (unless indicated otherwise), and aliquots were removed and plated on YPD at timed intervals and allowed to grow for several days at 30°C. For UV killing, cells were grown to log phase in YPD, plated on YPD, and irradiated (Stratagene UV Stratalinker 1800) with 0, 20, or 40 J/m² prior to incubation at 30°C.

Synchronization of cells in G1 phase

Strains were grown to log phase in YPD (pH 3.9), treated with 10 µg/ml α-factor for 1.5 hr, and supplemented with an additional 5 µg/ml α-factor for another 1.5 hr. Cells were then centrifuged and resuspended in YPD containing the 0.2 mM HU, 0.25 mM HU, 80 µg/ml benomyl, and/or 10 µg/ml nocodazole as indicated in the individual experiments.

Staining of cells for microtubule visualization

Cells were fixed by the addition of 5% formaldehyde to growing cultures and allowed to stand for at least 4 hr at 4°C. Cells were washed in PBS, and microtubules were immunostained using the antitubulin antibody YOL1/34 and a FITC-conjugated secondary antibody as described (Allen et al. 1994).
FACS analysis

The amount of 250 µl of cell culture (∼1.5 × 10^6 to 4 × 10^6 cells) was added directly to 1 ml of ethanol and allowed to stand 1 hr for fixation. Cells were washed once with 70% ethanol and once with FACS buffer (0.2 M Tris at pH 7.5, 20 mM EDTA). In a volume of 100 µl of FACS buffer, cells were treated with 1 mg/ml RNase A at 37°C for 2 hr. Cells were then washed in PBS, treated with 5 µg/ml propidium iodide in a final volume of 1 ml of PBS, and analyzed for fluorescence content using a Coulter: model Epics XL-MCL. The DNA content of ∼30,000 cells was determined for each sample.
α-Factor-arrested ρ0 intermediate strains were released into YPD containing 0.2% HU and 10 µg/ml nocodazole for 60 min; cells were spun down, washed, and resuspended in YPD containing 10 µg/ml nocodazole. Cells from different time points during and after HU treatment were fixed in 70% ethanol overnight. These were subsequently resuspended in 0.5 ml EDTA, 1.2 M sorbitol, and 1 M Tris (pH 7.5). Chromosome plugs were prepared following a rapid two-step protocol without use of proteinase K (Johnston 1994). Each 75 µl plug contained 4.5 × 106 cells. PFGE was carried out in a Bio-Rad DR II apparatus for 24 hr, at 200 V. Switching was done every 60 sec for the first 15 hr, and every 90 sec for the last 9 hr. Chromosomes were visualized with ethidium bromide. The gel was photographed and chromosome band intensities were quantitated using NIH Image software.

Strain and plasmid construction

The source of the MEC1 gene was pSAD3-3B, which is a 9.5-kb fragment of the MEC1 genomic locus cloned into pRS414 (Sikorski and Hieter 1989). pBAD45 contains the 7.7-kb SacI MEC1-containing fragment from pSAD3-3B cloned into the SacI site of pBAD40, which is a derivative of pRS416 (Sikorski and Hieter 1989) deleted between the NotI and SalI sites. pBAD54 is a GAP promoter expression vector made by cloning the GAP expression cassette, containing the GAP promoter and GAP terminator flanking a multicloning site, as a BamHI fragment from pAB23BXN into the BamHI site of YEpplac112 (Gietz and Sugino 1988).

The NRR1 and NRR3 ORFs were cloned by PCR and subcloned into pBS II KS(−) to make pBAD49 and pBAD58. The ends of each ORF were sequenced to verify lack of mutation, and the central parts of each ORF were replaced by the corresponding fragment from a functional genomic clone. For NRR1 this was a BstEII-XhoI fragment from pSE757 generating pBAD64, and for NRR3 it was a BstEII-HindIII fragment from pSE734 generating pBAD74. pBAD70 was made by subcloning the NRR1 ORF as a XhoI–NotI fragment from pBAD62 into XhoI–NotI-digested pBAD54, pBAD70 was made by subcloning the NRR3 ORF as a Ssp1406I(T4-filled-in)-NotI fragment from pBAD74 into pBAD54 that had been cut with XhoI and T4-filled in and subsequently cut again with NotI.

The RAD53 gene knockout has been described previously (Allen et al. 1994). MEC1 was knocked out by replacing a 7.5-kb BamHI fragment from pSAD3-3B with the BamHI fragment from pJA50 containing the H1S3 gene and a kanamycin resistance gene from Tn5 to form pWJ67. This removes all but the amino-terminal 33 amino acids from the MEC1 ORF. The 4.4-kb SacI fragment from pWJ67 containing the Δmecl::H1S3 deletion construct was transformed into Y323 to generate a diploid heterozygous for the mecl knockout Y617.

The TRP1-GAP–NRR1 expression cassette was created by subcloning a PsI–SacI fragment from pBAD70 into PsI–SacI-digested pRS404 to create pBAD114. rad53 and mec1 null mutants suppressed by this GAP–NRR1 expression cassette were generated as follows. pBAD114 was linearized within the TRP1 gene and transformed into Y312 and Y617 to create Y618 and Y619, and correct integration was confirmed by Southern blotting. Y618 was sporulated and Y606 and Y607 were recovered. Y619 was sporulated to obtain Y580 and Y581.

The temperature-sensitive orc2-1 mutant Y611 was generated by looping the orc2-1 allele from the URA3-integrating plasmid pJR1267 into Y 300. We then selected transformants for 5-FOA resistance and screened them for temperature sensitivity. Y612 was made by crossing Y611 with Y620 and sporulating and dissecting the resulting diploid Y621. Y613, Y614, Y615, and Y616 are four spores of identical genotype that were isolated from the diploid Y622, which was in turn created by a mating between Y602 and YCH266.

Y623 and Y624, His+ ρ0 derivatives of Y300 and Y301, respectively, were generated by serial culturing in minimal media containing ethidium bromide, as described in Fox et al. (1991).

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