RAD9 and DNA polymerase ε form parallel sensory branches for transducing the DNA damage checkpoint signal in Saccharomyces cerevisiae

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In response to DNA damage and replication blocks, yeast cells arrest at distinct points in the cell cycle and induce the transcription of genes whose products facilitate DNA repair. Examination of the inducibility of RNR3 in response to UV damage has revealed that the various checkpoint genes can be arranged in a pathway consistent with their requirement to arrest cells at different stages of the cell cycle. While RAD9, RAD24, and MEC3 are required to activate the DNA damage checkpoint when cells are in G1 or G2, POL2 is required to sense UV damage and replication blocks when cells are in S phase. The phosphorylation of the essential central transducer, Rad53p, is dependent on POL2 and RAD9 in response to UV damage, indicating that RAD53 functions downstream of both these genes. Mutants defective for both pathways are severely deficient in Rad53p phosphorylation and RNR3 induction and are significantly more sensitive to DNA damage and replication blocks than single mutants alone. These results show that POL2 and RAD9 function in parallel branches for sensing and transducing the UV DNA damage signal. Each of these pathways subsequently activates the central transducers Mec1p/Esr1p/Sad3p and Rad53p/Mec2p/Sad1p, which are required for both cell-cycle arrest and transcriptional responses.

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lethal mitosis, suggesting that cdc1 plays a role in stabilizing the S-phase state in response to replication blocks (Murakami and Okayama 1995). Furthermore, cell-cycle arrest in response to replication blocks requires the phosphorylation of cdc2 on tyrosine (Enoch and Nurse 1990).

In budding yeast, a number of genes have been identified that control the ability of cells to either arrest the cell cycle and/or activate the transcriptional response. These include RAD9, RAD17, RAD24, and MEC3, which are required for cell-cycle arrest in G1 (Siede et al. 1993, 1994) or G2 [Weinert and Hartwell 1988, 1993; Weinert et al. 1994] in response to DNA damage; MEC1/ SAD3/ESR1 and RAD53/SAD1/MEC2/SPO1, which are required for the S-phase checkpoint, the transcriptional response, and G1 and G2 arrest [Allen et al. 1994; Kato and Ogawa 1994; Weinert et al. 1994]; POL2/DUN2, which is required for the S-phase checkpoint [Navas et al. 1995]; and DUN1, which encodes a protein kinase that is activated in response to DNA damage and is necessary for the transcriptional response [Zhou and Elledge 1993]. MEC1 and RAD53 are central transducers of the signal. MEC1 belongs to the same subfamily of proteins as ATM, thereby highlighting the evolutionary conservation of this pathway [Greenwell et al. 1995; Morrow et al. 1995; Zakian 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]. It would stand to reason that genes that control only a subset of responses would act either upstream or downstream of both of these central elements. DUN1 is clearly downstream of RAD53 and is an effector of the transcriptional branch [Allen et al. 1994]. POL2 encodes DNA polymerase ε and is the best candidate for a sensor involved in this process. However, because pol2 mutants are specifically defective only for the response to replication blocks, Pol2p is unlikely to function outside of S phase when the replication complexes are not assembled. This predicts a different set of sensors/transducers involved in G1 and G2 control. The most likely candidates for such genes are the RAD9 group.

To date, no single model has emerged that describes the functional organization of these checkpoint genes in an ordered pathway. It is still not known whether there exist discrete but overlapping regulatory pathways triggered separately by unreplicated DNA and by DNA damage or whether genes such as the RAD9 group are simply additionally required to activate the DNA damage checkpoint in an otherwise identical signal transduction pathway. It has been shown that RAD9, MEC3, RAD24, and RAD17 play a role in processing DNA lesions and therefore could be involved in generating the signal to activate the checkpoint at G1/S and G2/M [Lydall and Weinert 1995]. However, it was not shown whether the effect on DNA processing was directly attributable to these proteins or attributable to defects in signaling that occur in these mutants. We have shown that RAD9 is not required for the modification of Rad53p in asynchronous cells in response to replication blocks and DNA damage [methyl methane sulfonate, {MMS}] [Sanchez et al. 1996], and it was proposed that RAD9 might function downstream of Rad53p [Carr 1996] or in an independent pathway.

To investigate signaling of DNA damage, we examined the ability of checkpoint mutants, alone or in combination, to phosphorylate Rad53p and activate transcription of the damage-inducible gene RNR3 in response to UV damage. We find that this response is dependent on two parallel sensory branches controlled by POL2 and RAD9 and propose a model whereby alternative sensory components of the checkpoint are used to both sense and transduce the DNA damage signal depending upon which stage of the cell cycle the damage was incurred.

**Results**

**RNR3 activation in asynchronous and G1-arrested cells**

DNA polymerase ε, POL2, has been implicated in DNA damage signaling in S-phase cells. However, it seemed unlikely that POL2 would be involved outside of S phase, for example, in G1, when the replication apparatus was not fully functional. To search for the signaling molecules that function outside of S phase, we first examined the ability of G1 and asynchronous cells to activate transcription of the damage-inducible gene RNR3 in response to UV irradiation. RNR3 is a downstream effector for both the DNA damage and S-phase checkpoint pathways and its induction parallels cell-cycle delay in pathways that involve POL2 and RAD9 and is dependent on RAD53 (Elledge et al. 1993; Allen et al. 1994; Navas et al. 1995). Unlike asynchronous cultures, yeast cells arrested in G1 by α-factor are unable to activate transcription of RNR3 in response to UV light. However, by irradiating G1-arrested cells and then releasing the arrest (G1-S), RNR3 induction could be detected in wild-type cells [Fig. 1A]. Whereas asynchronous rad9 mutants show a significant induction of RNR3, the induction when damaged in G1 is dependent on RAD9. This effect could be attributable to either reduced inducibility or altered kinetics of induction. To examine this, a time course for RNR3 induction was performed using the G1, damage and release (G1-S) protocol. Although the timing of maximal induction for rad9 was delayed by 30 min compared with WT, the maximal induction was only twofold compared with ~13-fold in WT cells [Fig. 1B], thus disproving the kinetics hypothesis. Similar results were obtained for RNR2 induction [data not shown]. It is not known whether RNR3 is cell cycle-regulated. However, because RNR3 has several MCB and SCB elements in its promoter [Elledge et al. 1992], absence of inducibility of RNR3 might be attributable to an absence of transcription factors capable of activating RNR3 transcription at START. Alternatively, part of the signal transduction machinery might be inactive at the α-factor block. To examine this, we tested whether the last step in the signaling pathway, Dunlp, was functional in G1-
arrested cells. Dun1p kinase activity was examined in α-factor arrested cells or after passage through START using the G₁–S protocol (Fig. 1C). Dun1p kinase was not activated in α-factor-arrested cells but was activated after START, indicating that this part of the signal transduction pathway is likely to be inactive in α-factor-arrested cells.

RNR3 induction in the G₁–S protocol is measured in cells irradiated at the α-factor arrest but collected at a later stage of the cell cycle (late G₁ or S). It is possible that the requirement for RAD9 for RNR3 induction is dependent not on the original cell-cycle stage where the damage was incurred, but upon subsequent entry into later stages. To determine whether the G₁–S protocol is indeed measuring RNR3 induction in G₁ as opposed to S phase, cdc4, cdc4rad9, cdc7, and cdc7rad9 cells were synchronized with α-factor at the permissive temperature, irradiated with UV, and then released into the non-permissive temperature before assaying for RNR3 transcription (Fig. 1D). Both CDC4 and CDC7 execution points occur after START but before S-phase entry (Hermanford and Hartwell 1974; Hollingsworth and Selafani

Figure 1. (See facing page for legend.)
1990; Smith et al. 1992). RNR3 is highly induced in cells that were irradiated in G1 and subsequently blocked at either of these points. In both cases, the induction was also found to be dependent on RAD9, thus indicating that the G1-S protocol measures RNR3 induction in G1 and is not dependent upon S-phase entry.

pol2 rad9 double mutants are defective for RNR3 induction in cycling cells

Because POL2 has been implicated as a potential sensor of DNA damage, S-phase checkpoint-defective pol2 mutants were examined for RNR3 induction by UV irradiation. pol2 mutants were found to have greatly reduced RNR3 inducibility in asynchronous cultures (Fig. 2). Because of its replication defect, ~90% of pol2 cells in an asynchronous population were found to be in S phase (data not shown). The residual induction may therefore be attributable to the small population of cells that are in G1 or G2, and it is possible that the POL2 pathway may be the major sensor of UV damage during S phase. G1-synchronized pol2 mutants showed an inducibility similar to WT, indicating that POL2 is not required for the transcriptional response in G1. This is consistent with the observation that POL2 does not have a role in arresting the cell cycle in G1 (Navas et al. 1995).

The RNR3 induction phenotype seen in pol2 mutants therefore is complementary to that observed in rad9 cells. Furthermore, pol2 rad9 double mutants are completely defective in RNR3 induction by UV light both in cycling and G1-synchronized cells. This response is similar to that seen in rad53 and mec1 mutants (see below, Fig. 5B) indicating that pol2 rad9 cells are unable to activate the checkpoint at all stages of the cell cycle in response to UV damage. If POL2 functions in S phase, rad9 mutants treated with UV-light using the G1-S protocol might be expected to enter S phase and activate the POL2-dependent pathway to induce RNR3. RNR3 is not induced when rad9 mutants are damaged in G1. Perhaps UV lesions occurring in G1 are repaired prior to entry into S phase or are metabolized by a repair pathway that does not generate a signal. Nevertheless, these results suggest that the POL2 and RAD9 pathways function primarily in different cell-cycle stages and are the primary sensors and transducers of the cell-cycle checkpoint information generated by UV damage.

Figure 1.  The transcriptional induction of RNR3 is dependent on RAD9 in cells damaged in G1 and released into the cell cycle. [A] Northern blot analysis of total RNA isolated from isogenic wild-type (TWY397) or rad9 [TWY398] strains. Cells were grown in YPD and treated with (+) or without (−) 80 J/m² UV light. [Asy] Cells grown asynchronously. [G1] Cells arrested with α-factor and maintained in G1 through the course of the experiment. [G1-S] Cells arrested with α-factor, UV-irradiated, then released from the α-factor block for 1.5 hr at 24°C. [G1-S] mRNA was visualized on Northern blots using RNR3 and actin as probes. Bar graphs show the levels of RNR3 mRNA relative to actin mRNA in each lane, arbitrary units.

Figure 2.  pol2 rad9 is defective for RNR3 induction by UV. Northern blot analysis of total RNA isolated from cells untreated [hatched bars] or treated [solid bars] with 80 J/m² UV-irradiation. Strains used were isogenic wild-type (Y300), rad9 [Y438], pol2 [Y439] and pol2 rad9 [Y440] grown either asynchronously (Asy) or α-factor-arrested populations followed by release from the α-factor block for 1.5 hr at 24°C (G1-S). mRNA was visualized on Northern blots using RNR3 and actin as probes. Bar graphs show the levels of RNR3 mRNA relative to actin mRNA in each lane, arbitrary units.
POL2 and RAD9 are required to transduce the DNA damage signal at different stages of the cell cycle

To explore further the complementary roles of POL2 and RAD9 in transducing the damage signal in different cell-cycle stages, wild-type, rad9, pol2, and pol2rad9 cells were synchronized in G1 with α-factor, released from the block, and then irradiated with UV at different times after release (Fig. 3). To correlate the magnitude of RNR3 induction with a particular stage of the cell cycle, the position of the cells in each time point were monitored by FACS analysis both at the time of irradiation and 1 hr later, when the cells were harvested for RNR3 transcription. As shown earlier, the induction of RNR3 in wild-type cells is high when irradiated at the α-factor arrest stage. The induction then consistently diminishes about 2.5-fold when cells are irradiated during S phase (30–45 min after α-factor release) and then increases slightly when cells are irradiated as they enter G2 (60 min) and decreases as they enter mitosis. rad9 cells are initially uninducible when irradiated in G1 but the induction increases and approaches levels similar to that of wild-type when rad9 cells enter or traverse through S phase. As cells enter G2, however, rad9 cells are once again uninducible relative to wild-type. Thus, it is mainly in G1 or G2 where RAD9 appears to play a major role in trans-
Parallel sensory branches for DNA damage signaling

Rad53p modification in response to UV damage is dependent on both POL2 and RAD9

If POL2 and RAD9 function as parallel sensors, then the modification of a downstream central transducer should be dependent on these two genes in a manner analogous to RNR3 induction. RAD53 controls both cell-cycle arrest and transcriptional induction in response to DNA damage and replication blocks. Rad53p is a cell-cycle regulated protein, whose abundance increases during S/G2, and is modified by phosphorylation in response to DNA-damaging agents and replication blocks [Sanchez et al. 1996; Sun et al. 1996]. We examined Rad53p modification in wild-type, rad9, pol2, and pol2rad9 mutants. The modification of Rad53p was concordant with RNR3 induction. Whereas Rad53p was modified in both asynchronous rad9 and pol2 cells in response to UV, it showed no detectable modification in pol2rad9 double mutants [Fig. 4A]. These results indicate that RAD9 and POL2 function upstream of RAD53 in the UV response pathway. Moreover, in cells that were irradiated in G1 and then released [Fig. 4B], a marked decrease in the modification of Rad53p was seen in rad9 but not pol2 cells, a result that parallels the RNR3 transcription results [Fig. 2].

Transcriptional response of other checkpoint mutants shows roles consistent with their requirement for cell-cycle arrest at various stages of the cell cycle

Recently, RAD9, RAD17, RAD24, and MEC3 have been postulated to be involved in processing cdc13-induced lesions near the telomeres [Garvik et al. 1995; Lydall and Weinert 1995]. Although RAD9 was shown to act differently from RAD24, MEC3, and RAD17 in processing DNA damage, all four genes appear to act in the same pathway with respect to cell-cycle arrest and would be expected to have phenotypes similar to rad9 mutants in the RNR3 transcription assay. Both mec3 and rad24 mutants behave like rad9 in this assay [Fig. 5A], indicating that all three genes are involved in transducing the UV signal in late G1, rad17 mutants were not tested. Mutants in mec1 and rad53 were also defective in UV-induced RNR3 expression in all stages of the cell cycle [Fig. 5B]. Similar results for mec1 and rad53 mutants recently have been obtained by Kiser and Weinert [1996]. However, rad9, rad24, and mec3 are proficient in RNR3 induction in response to hydroxyurea (HU) [Fig. 5C; Elledge and Davis 1990], unlike pol2, mec1, and rad53 mutants.

cdc13 cells exhibit RAD9-dependent arrest in late S or early G2 at 36°C because of the generation of telomere-

producing the damage signal in wild-type cells. It is not clear whether the slight increase in inducibility at later times relative to wild-type cells is significant because cells are losing synchrony at this point.

pol2 cells have high levels of induction when irradiated in G1, with α-factor and the induction subsequently diminishes as the cells enter and are irradiated in S phase. pol2 cells have highly elongated S phases [Navas et al. 1995]; therefore, the cells fail to progress to G2 during the time course of this experiment. The result, however, clearly shows that POL2 plays a significant role in transducing the damage signal when cells are irradiated during S phase. Furthermore, pol2rad9 cells were found to be uninducible during the entire course of the experiment, demonstrating that the ability of these cells to transduce the damage signal in all stages of the cell cycle has been impaired greatly.

Figure 4. Regulation of phosphorylation of Rad53p by RAD9 and POL2. Cells were treated as in Fig. 2 and protein extracts were prepared, separated by SDS–PAGE, and immunoblotted with antibodies to Rad53p [Sanchez et al. 1996]. [A] Lanes 1, 2 contain extracts from asynchronous cultures of the RAD53 deletion strain (Y677) rescued by a plasmid containing a high copy suppressor. Lanes 3, 4, 9 contain extracts from parallel asynchronous cultures of wild-type cells (Y300) that were untreated [lanes 3, 9] or irradiated with 80 J/m² of UV [lane 4] and incubated at 30°C for 1.5 hr. Lanes 5, 6 contain extracts from rad9 (Y438) cells untreated [lane 5] or treated [lane 6] with UV. Lanes 7, 8 contain extracts from pol2 (Y439) and pol2rad9 (Y440) cells after 80 J/m² of UV irradiation. [B] α-factor-arrested cells were irradiated followed by release from the α-factor block (G1-S) and incubated for 1 hr prior to preparation of extracts. Lane 1 contains extract from Δrad53 strain, lanes 2, 3: wild-type cells (Y300), lanes 4–7 rad9 (Y438), pol2 (Y439) and pol2rad9 (Y440) after UV irradiation, respectively. Proteins were detected by enhanced chemiluminescence. Arrows refer to the different forms of Rad53p.
proximal ssDNA that is thought to be recognized as DNA damage by the cell (Garvik et al. 1995). To demonstrate the requirement for RAD9 in transducing the checkpoint response at G₂, we assayed RNR3 induction in wild-type, cdc13, and cdc13rad9 cells that were synchronized in G₁ at 24°C and then released from the block at the nonpermissive temperature. We found the induction of RNR3 in cdc13 cells at 36°C to be dependent on RAD9 (Fig. 5D) and MEC3 (data not shown), demonstrating further the involvement of the RAD9 group in the generation or transduction of checkpoint signals at G₂.

double mutants are more sensitive to UV and HU than either mutant alone

These data suggest that the RAD9 group and POL2 have independent but parallel functions with respect to transduction of the DNA damage signal and should be in different epistasis groups. Consistent with this hypothesis, pol2rad9 double mutants were found to be more sensitive to killing by UV than either single mutant, indicating independent functions at the semipermissive temperature of 28°C for the pol2 allele (Fig. 6A). Previously, it has been reported that pol2 mutants are checkpoint-defective at the permissive temperature (Navas et al. 1995). However, we found that the conditions of those experiments actually allowed the temperature to increase above room temperature (T. Navas, unpubl.). Subsequently, we have found that the HU-induced lethality of these mutants is exacerbated at higher temperatures (28°C or 30°C), indicating that pol2 may still have some residual checkpoint activity at 24°C. Although POL2 and RAD9 function separately in transducing the damage signal, the increased sensitivities of the double mutants could also reflect an additional independent function distinct from their signaling roles. For example, distinct roles in DNA repair could produce this synergistic effect.

double mutants also showed increased sensitivity to HU compared with either single mutants (Fig. 6B). Because rad9 mutants show normal signaling for HU-induced RNR3 expression and are not themselves sensitive to HU (Elledge and Davis 1990), it was anticipated that rad9 mutants would have no effect on HU-sensitivity of pol2 mutants. Surprisingly, rad9 has a pronounced effect indicating that RAD9 may have a minor role in the response to HU arrest during S phase that is not detectable until the major pathway is knocked out.
RAD53 overexpression partially suppresses the HU sensitivity of pol2rad9 double mutants

MEC1 and RAD53 are required for all checkpoint and RNR3 transcriptional responses to DNA damage, whereas pol2 and rad9 mutants display defects in only a subset of these responses. POL2 and RAD9 must either transduce a subset of signals to MEC1 and RAD53 or act downstream to contact only a subset of effectors. We have shown previously that overexpression of RAD53 could partially suppress the sensitivity of rad9 (Allen et al. 1994) mutants to DNA-damaging agents such as UV, suggesting that RAD9 may function upstream of RAD53. Overexpression of RAD53 may enhance its role in checkpoint-mediated arrest and its ability to suppress may represent a reduced requirement for upstream gene function. Support for this ordering comes from the fact that Rad53p modification by UV damage is dependent upon both RAD9 and POL2 (Fig. 4) and argues that both RAD9 and POL2 are upstream of RAD53. Furthermore, we have observed that the overexpression of RAD53 can also partially suppress the HU sensitivity of pol2 (data not shown) and pol2rad9 mutants (Fig. 6B). These results indicate that the suppression of pol2 and rad9 mutant phenotypes by RAD53 overexpression is likely to mimic its normal function in promoting checkpoint arrest.

Discussion

RAD9 was the first of the checkpoint genes to be identified in yeast and has been hypothesized to directly mediate cell-cycle arrest (Weinert and Hartwell 1988; Hartwell and Weinert 1989). Here we provide evidence that RAD9 is involved in the signal sensing and transducing branch of the pathway, consistent with its recently proposed role in processing of DNA damage for repair (Lydall and Weinert 1995). Siede et al. (1994) have shown the absence of G1 delay for rad9 and rad24 cells when damaged with UV, and Weinert et al. (1994) have shown a G2 checkpoint defect for rad9 mutants both by UV and by cdc13 loss of function. We have shown that rad9 mutants are uninducible for RNR3 and fail to modify Rad53p when damaged at G1 or G2, consistent with the hypothesis that a RAD9-dependent pathway is the primary transducer of UV damage in G1 and G2. pol2 mutants are known to delay progression at G1 and G2, when damaged with UV and are proficient for RNR3 at these points. pol2 mutants that are defective for the S-phase checkpoint show a reduction of UV-induced RNR3 transcription and Rad53p phosphorylation when the majority of the cells are in S phase, consistent with the hypothesis that a POL2-dependent pathway is the primary transducer of UV damage in S phase. The defect in Rad53p phosphorylation asynchronously grown pol2 mutants was not as severe as that observed in rad9 mutants; however, Rad53p modification was completely absent in rad9pol2 double mutants, indicating the complete dependence on these two genes for damage signaling.

Currently it is unknown whether the RAD9 group of gene products or additional proteins act as the actual sensors in this pathway. The RAD17 gene, which belongs to this group, is homologous to both S. pombe rad1* and to Ustilago maydis REC1 (Long et al. 1994;
Figure 7. A model depicting the arrangement of checkpoint genes within the DNA damage and S-phase checkpoint pathway. When DNA is damaged in G1, RNR3 is transcriptionally activated after passage through START and this induction is dependent upon RAD9, RAD24, MEC3 and possibly RAD17. A similar series of requirements are observed when cells are damaged in G2, based upon cdc13-mediated RNR3 induction and the dependency for UV-induced cell-cycle arrest in G2. POL2 recognizes damage when cells are in S phase. Both sensory branches transduce the damage signal in a MEC1/SAD3/ESR1-dependent manner to activate RAD53/SAD1/MEC2. MEC1 and RAD53 control both cell-cycle arrest and the DUN1-dependent transcriptional induction of damage-inducible genes such as RNR3 in all phases of the cell cycle. RAD9 may play a minor role during S phase but is not shown on that branch in this figure.

Siede et al. 1996). REC1 encodes a 3’–5’ exonuclease implicating this protein in DNA lesion processing [Thelen et al. 1994; Onel et al. 1996]. In this respect the RAD9 group may be similar to the recBC pathway in Escherichia coli known to process double-stranded breaks to produce ssDNA for sensing by recA [Walker 1985] and is therefore indirectly involved in DNA damage signaling. The fact that RAD9 is upstream of RAD53 lends further support for the hypothesis that the RAD9 group of genes function in directly processing the lesions.

POL2 and RAD9 constitute two parallel sensory branches of the DNA damage checkpoint

The data presented here are consistent with a model shown in Figure 7 in which the RAD9 group of genes operates as the primary signal sensors/transducers controlling the UV-activated DNA damage response outside of S phase. Once DNA replication is initiated, DNA polymerase ε becomes the primary sensor of UV damage or processed UV lesions. Thus POL2 and the RAD9 group are parallel components of the sensory branches in the DNA damage response pathway. An intriguing question is, Why would the RAD9 pathway be less involved in sensing or processing DNA damage during S phase? One possibility is that the RAD9 pathway might be partially inactivated during S phase because it might recognize replication structures as DNA damage. Because one aspect of the response to DNA damage during S phase is a DNA replication block or slowdown (Paulovich and Hartwell 1995), it would be deleterious to the cell to activate this pathway during a normal S phase. Another possibility is that UV lesions that normally result in activation of the RAD9 pathway are metabolized differently during S phase such that they are not well recognized by the RAD9 pathway, which has also been suggested by Paulovich and Hartwell (1995). This may also reflect an incompatibility between different types of sensors that recognize damaged DNA substrates. For instance, POL2 may recognize damage within the context of a replication fork that meets a damaged site in the process of DNA replication. Perhaps once damage is encountered at a fork, it is no longer accessible to the RAD9 pathway. Alternatively, perhaps, RAD9 can still process damage but no longer signals during S phase. It is also possible that the signal threshold required for arrest is higher in S phase than in G1 and G2 and that the POL2 pathway generates a stronger signal than the RAD9 pathway. This would be consistent with a minor role in S-phase signaling for RAD9 and its synthetic HU-sensitive phenotype when combined with pol2 mutations.

Different sensory branches activate common central transducers of the checkpoint

As independent components of the sensory branches of the checkpoint, POL2 and RAD9 should therefore function upstream of the common central transducers MEC1 and RAD53. Previous reports, however, have shown that Rad53p is modified in asynchronous rad9 cells when treated with HU or MMS [Sanchez et al. 1996; Sun et al. 1996]. This led to the suggestion that RAD9 may either function downstream of RAD53 or may be involved in a pathway independent of RAD53. However, both HU and MMS have been shown to block or slow down DNA replication in cells [Paulovich and Hartwell 1995] and may therefore activate the S-phase checkpoint and signal through Pol2p in a RAD9-independent manner. If RAD9 functions downstream of RAD53 in a linear pathway, then UV damage would not be expected to induce RNR3 in asynchronous cultures of rad9 cells because asynchronous mec1 and rad53 cells themselves have been shown to be uninducible [Fig. 5B; Allen et al. 1994]. Moreover, the fact that both RNR3 induction and Rad53p modification in response to UV is dependent on RAD9 when cells are damaged in G1 demonstrates that RAD9 functions upstream of RAD53. The cdc13-mediated RNR3 induction in G2 is RAD9-dependent. Both events require the activation of Rad53p, which has also been shown to be dependent on MEC3, a member of the RAD9 group of genes [Sun et al. 1996]. Because it has also been shown that MEC1 functions upstream of RAD53, MEC1 is more likely to be the immediate target for activation by the different sensory branches of the checkpoint.

Checkpoints and cancer

It remains to be seen whether other types of DNA dam-
age in addition to UV photoproducts are sensed through the RAD9 and POL2 pathways. However, because it is clear that many of the cell-cycle checkpoint genes are conserved throughout evolution, the parallel nature of the signaling branches are also likely to be conserved. The existence of parallel branches for sensing and transducing the UV DNA damage signal in eukaryotes has certain implications for efficacy of chemotherapeutic agents in the treatment of cancer. Cells from many different cancer types show defects in cell-cycle checkpoints to some degree. This often distinguishes them from the surrounding normal tissues. If it were possible to eliminate a parallel pathway in these cells through specific drugs, the cancer cells should become profoundly sensitive to DNA damage relative to the normal cells that would have one of the pathways operational. This would mimic the situation in the pol2rad9 double mutants relative to either single mutant. This enhanced sensitivity might then be exploited to specifically eliminate the malignant cells.

Materials and methods

Yeast strains

Yeast strains used in this study are listed in Table 1. Y438 (rad9Δ::HIS) was created by transforming Y300 with NotI-cleaved pTW033 and selecting for His+ prototrophy. Correct transformants were checked for UV sensitivity and by Southern blot analysis. Y439 (pol2-12) was created by transforming Y300 with BglII-cleaved pAN44 (TRP1, pol2-12) and selecting for Trp+ prototrophy, Ts- and HU- sensitivity at 100 mM HU. BglII cleaves within the POL2 gene. This particular allele survives at 30°C in the Y300 background, unlike its original parent strain, TC102 pol2-12. Y440 (pol2rad9) was created by transforming Y438 harboring RAD9 on a CEN URA plasmid, with BglII-restricted pAN44 (Navas et al. 1995). Ura- transformants were selected on 5-fluoro-orotic acid (5-FOA) and checked for UV sensitivity, Ts- and HU-sensitivity phenotypes. Southern blot analysis was performed to confirm correct integration in the double mutant.

UV induction in asynchronous and α-factor synchronized cells

Asynchronous wild-type or mutant cells (OD600 = 0.4) were typically spread on YPD plates and irradiated with 80 J/m2 UV using a Stratalinker (Stratagene). Cells were scraped off the plates, washed once with water and then resuspended back in liquid nitrogen. It was found that RNR3 is maximally induced in cells 1.5 hr after treatment with UV at 24°C. For G1 or G1-S cultures, cells were synchronized in YPD (pH 3.9) with 15 μg/ml of α-factor (Calbiochem) for 3 hr at 24°C before UV and were resuspended in YPD (pH 3.9) and additionally incubated with fresh α-factor (10 μg/ml) for G2 cultures, whereas G2-S cultures were washed once with water and resuspended in YPD for 1 hr before harvesting and quick freezing in liquid nitrogen.

Table 1. Yeast strains used in this study

| Strain     | Genotype                                    | Source                      |
|------------|---------------------------------------------|-----------------------------|
| TWY397     | MATα ura3 his7 leu2 trp1                     |                             |
| TWY398     | MATα rad9Δ::LEU2 his7 ura3 leu2 trp1         |                             |
| TWY308     | MATα mec1-1 ura3 trp1                        |                             |
| TWY312     | MATα mec2-1 ura3 his7 trp1                   |                             |
| TWY316     | MATα mec3-1 ura3 his3 trp1                   |                             |
| TWY399     | MATα rad24-1 ura3 his7 leu2 trp1             |                             |
| TWY431     | MATα cdc13-1 ura3 his3                      |                             |
| TWY72      | MATα cdc13-1 rad9Δ::LEU2 his7 trp1 ura3 can100 | T. Weinert (University of Arizona, Tucson) |
| Y300       | MATα can1-100 ade2-1 his3-11.15 leu2-3,112 trp1-1 ura3-1 | Allen et al. 1994 |
| Y321       | Y300 + pUN70 [URA3]                         |                             |
| Y301       | MATα sad1-1 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Allen et al. 1994 |
| Y306       | MATα sad3-1 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Allen et al. 1994 |
| WS9110-3D  | MATα cdc4-1 ade2-1 ura3-52                   |                             |
| WS9110-3D rad9Δ | MATα cdc4-1 rad9Δ::URA3 ade2-1 ura3-52     |                             |
| WS9120-10A | MATα cdc7-1 ura3-52                         |                             |
| WS9120-10A rad9Δ | MATα cdc7-1 rad9Δ::URA3 ura3-52            |                             |
| Y438       | MATα rad9::HIS3 can1-100 ade2-1 his3-11.15 leu2-3,112 trp1-1 ura3-1 | This study |
| Y439       | MATα can1-100 ade2-1 his3-11.15 leu2-3,112 trp1-1 ura3-1 pol2-12-TRP | This study |
| Y440       | MATα rad9::HIS3 can1-100 ade2-1 his3-11.15 leu2-3,112 trp1-1 ura3-1 pol2-12-TRP1 | This study |
| Y441       | Y300 + pZZ74 [GAP-HA DUN1 2μ URA3]          |                             |
| Y442       | Y300 + pAB23BXN [2μ URA3]                   |                             |
| Y443       | Y440 + pJA98 [GAL-RAD53 URA3]               |                             |
| Y444       | Y440 + pUN70 [URA3]                        |                             |
**Northern blot analysis and flow cytometry**

Total RNA was isolated using the hot-acid-phenol method [Kohrer and Domdey 1991], and Northern blot analysis was performed using formaldehyde-1% agarose gels [Sambrook et al. 1989]. The 2.5-kb MluI–HindIII fragment of RNR3 from pSE734 was used as probe. Radioactivity was quantitated using a PhosphorImager [Molecular Dynamics] and then normalized using the actin gene as loading control. FACS analysis was performed as described previously [Allen et al. 1994].

**Dun1 kinase assay and modification of Rad53p**

Wild-type cells (Y300) containing pZZ74 (DUN1 expressed from the GAP promoter) or pAB32RXN (vector) were synchronized in G1 with α-factor and treated with or without 80 J/m² UV-irradiation and either maintained in G0 or released from the GI block (G1–S) for 1 hr after irradiation. Dun1 was immuno-precipitated from 200 µg of yeast protein and peptide kinase assays were performed with the peptide LKKLTRRASFSGQ as described [Pearson et al. 1993]. Western analysis was performed using the ECL system [Amersham]. The modification of Rad53p by UV in wild-type and mutant cells was determined as described previously [Sanchez et al. 1996].

**Determination of the UV sensitivity of pol2rad9 at the semipermissive temperature**

Mid-log phase cultures of wild-type (Y300), rad9 (Y438), pol2 [Y439], and pol2rad9 (Y440) that were grown overnight in minimal media at 24°C were incubated at 28°C for 3 hr, spread to about 1000 cells on SC plates auxotrophic for the particular mutant and then irradiated with increasing doses of UV using a Stratalinker. The plates were then incubated at 28°C and rate of survival was determined.

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Friedberg, E.C., G.C. Walker, and W. Siede. 1995. DNA Repair and Mutagenesis. American Society for Microbiology, Washington, DC.

Garvik B., M. Carson, and L. Hartwell. 1995. Single-stranded DNA arising at telomeres in cdcl3 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell Biol. 15: 6128–6138.

Greenwell P.W., S.L. Kronmal, S.E. Porter, J. Gassenhuber, B. Obermaier, and T.D. Petes. 1995. TEL1, a gene involved in controlling telomere length in S. cerevisiae, is homologous to the human ataxia telangiectasia gene. Cell 82: 829–839.

Hartwell, L.H. and T.A. Weinert. 1989. Checkpoint: Controls that ensure the order of cell cycle events. Science 246: 629–634.

Hereford, L.M. and L.H. Hartwell. 1974. Sequential gene function in the initiation of saccharomyces cerevisiae DNA synthesis. J. Mol. Biol. 84: 445–461.

Hollingsworth, R.E. and R.A. Sclafani. 1990. DNA metabolism gene CDC7 from yeast encodes a serine(threonine) protein kinase. Proc. Natl. Acad. Sci. 87: 6272–6276.

Kastan, M.B., Q. Zhan, W.S. El-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plunkett, B. Volgestein, and A.J. Fournace, Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in Ataxia-Telangiectasia. Cell 71: 587–597.

Kato, R. and H. Ogawa. 1994. An essential gene, ESR1, is required for mitotic cell growth, DNA repair and meiotic recombination in Saccharomyces cerevisiae. Nucleic Acids Res. 22: 3104–3112.

Kiser, G. and T.A. Weinert. 1996. Distinct roles of yeast MEC and RAD checkpoint genes in transcriptional induction after DNA damage and implications for function. Mol. Biol. Cell. 7: 703–718.

Kohrer, K. and H. Domdey. 1991. Preparation of high molecular weight RNA. Methods Enzymol. 194: 398–405.

Lehmann, A.R. and A.M. Carr. 1995. The ataxia-telangiectasia gene: A link between checkpoint controls, neuro degenera-
Parallel sensory branches for DNA damage signaling

SCM4, a gene that suppresses mutant cdc4 function in budding yeast. *Mol. Gen. Genet.* 235: 285–291.

Sun, Z., D.S. Fay, F. Marini, M. Foiami, and D.F. Stern. 1996. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. *Genes & Dev.* 10: 395–406.

Thelen, M.P., K. Onel, and W.K. Holloman. 1994. The *REC1* gene of *Ustilago maydis* involved in the cellular response to DNA damage encodes an exonuclease. *J. Biol. Chem.* 269: 747–754.

Walker, G.C. 1985. Inducible DNA repair systems. *Annu. Rev. Biochem.* 54: 425–457.

Walworth, N. and R. Bernards. 1996. *rad*-dependent response of the *chk1*-encoded protein kinase at the DNA damage checkpoint. *Science* 271: 353–356.

Walworth, N., S. Davey, and D. Beach. 1993. Fission yeast chk1 protein kinase links the rad checkpoint pathway to cdc2. *Nature* 363: 368–371.

Weinert, T.A. and L.H. Hartwell. 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* 241: 317–322.

—. 1993. Cell cycle arrest of cdc mutants and specificity of the *RAD9* checkpoint. *Genetics* 134: 63–80.

Weinert, T.A., G.L. Kiser, and L.H. Hartwell. 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes & Dev.* 8: 652–665.

Zakian, V.A. 1995. *ATM*-related genes: What do they tell us about functions of the human gene. *Cell* 82: 685–687.

Zhou, Z. and S.J. Elledge. 1993. *DUN1* encodes a protein kinase that controls the DNA damage response in yeast. *Cell* 75: 1119–1127.
RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in Saccharomyces cerevisiae.

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