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

Tony A. Navas, Yolanda Sanchez, and Stephen J. Elledge¹

Verna and Mars McLean Department of Biochemistry, Department of Molecular and Human Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030 USA

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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In¹ response to DNA damage and replication blocks, eukaryotic cells arrest the cell cycle and induce the transcription of genes that facilitate DNA repair processes. Under certain circumstances they may activate a programmed cell death pathway. The pathways that control these responses are being explored in several organisms. In mammals, the ability to sense and respond to DNA damage is critical to their long-term survival. Mutations in genes such as p53 or *ATM* (ataxia telangiectasia mutated) that control these processes result in a predisposition to cancer attributable to increased genomic instability and mutagenesis (Enoch and Norbury 1995). The *ATM* gene is a member of the lipid kinase family of proteins and is a likely transducer of a DNA damage signal (Lehmann and Carr 1995; Savitsky et al. 1995; Zakian 1995). p53 is a transcription factor that is activated in response to DNA damage and that controls the G₁ DNA damage checkpoint (Kastan et al. 1992; Lu and Lane 1993). 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).

Mutants defective for the DNA damage checkpoint and/or the replication checkpoint have also been isolated in *Schizosaccharomyces pombe* (Al-Khodairy and Carr 1992; Enoch et al. 1992; Al-Khodairy et al. 1994). The bulk of the checkpoint mutants, including *rad1*, *rad3*, *rad9*, *rad17*, *rad26*, and *hus1*, are defective for both checkpoint pathways indicating that the DNA damage and the S-phase checkpoint pathways share overlapping regulatory components (reviewed in Sheldrick and Carr 1993). Whereas many genes are shared in the two responses, there are genes that are specifically involved in one pathway only. The fission yeast protein kinase *chk1*⁺ and 14-3-3 homologs *rad24*⁺ and *rad25*⁺ (Walworth et al. 1993; Ford et al. 1994; Carr et al. 1995) appear to be specific for the DNA damage checkpoint. The activation of *chk1*⁺ was shown to be dependent on the activity of the *ATM* homolog *rad3*⁺ (Walworth and Bernards 1996). Other genes, such as *cds1*⁺, are not required for the DNA damage checkpoint, but have been shown to be required for the response to replication blocks. *cds1* mutants do not enter mitosis in the presence of replication blocks, but after removal of the block, they enter a

¹Corresponding author.

lethal mitosis, suggesting that *cds1* 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 G_1 (Siede et al. 1993, 1994) or G_2 (Weinert and Hartwell 1988, 1993; Weinert et al. 1994) in response to DNA damage; *MEC1/SAD3/ESR1* and *RAD53/SAD1/MEC2/SPK1*, which are required for the S-phase checkpoint, the transcriptional response, and G_1 and G_2 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 G_1 and G_2 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 G_1/S and G_2/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 G_1 -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 G_1 , 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 G_1 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 G_1 by α -factor are unable to activate transcription of *RNR3* in response to UV light. However, by irradiating G_1 -arrested cells and then releasing the arrest (G_1-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 G_1 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 G_1 -damage and release (G_1-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, Dun1p, was functional in G_1 -

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 (Hereford and Hartwell 1974; Hollingsworth and Sclafani

Figure 1. (See facing page for legend.)

1990; Smith et al. 1992). *RNR3* is highly induced in cells that were irradiated in G_1 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 G_1 -S protocol measures *RNR3* induction in G_1 and is not dependent upon S-phase entry.

pol2rad9 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 G_1 or G_2 and it is possible that the *POL2* pathway may be the major sensor of UV damage during S phase. G_1 -synchronized *pol2* mutants showed an inducibility similar to WT, indicating that *POL2* is not required for the transcriptional response in G_1 . This is consistent with the observation that *POL2* does not have a role in arresting the cell cycle in G_1 (Navas et al. 1995).

The *RNR3* induction phenotype seen in *pol2* mutants therefore is complementary to that observed in *rad9* cells. Furthermore, *pol2rad9* double mutants are completely defective in *RNR3* induction by UV light both in cycling and G_1 -synchronized cells. This response is similar to that seen in *rad53* and *mec1* mutants (see below, Fig. 5B) indicating that *pol2rad9* 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 G_1 -S protocol might be expected to enter S phase and activate the

Figure 2. *pol2rad9* 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 *pol2rad9* (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 (G_1 -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-dependent pathway to induce *RNR3*. *RNR3* is not induced when *rad9* mutants are damaged in G_1 . Perhaps UV lesions occurring in G_1 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 G_1 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. (G_1) Cells arrested with α -factor and maintained in G_1 through the course of the experiment. (G_1 -S) Cells arrested with α -factor, UV-irradiated, then released from the α -factor block for 1.5 hr prior to harvesting. FACS profiles are shown for cells from asynchronous populations, α factor-synchronized populations at the point of UV irradiation (0 hr) and 1.5 hr after UV irradiation when total RNA was isolated. Identical results were obtained with strains of a different background such as Y300. (B) A time course of *RNR3* induction following UV irradiation. RNA was prepared at 30 min intervals from isogenic wild-type (Y300; solid bars) and *rad9* cells (Y438; hatched bars) cultures arrested with α -factor, irradiated with 80 J/m² UV light, and released from the α -factor block at 30°C. At bottom, the fold induction of *RNR3* normalized to the actin-loading control. The ratio of the $t=0$ sample was assigned the value of 1. (C) The effects of UV-irradiation on Dun1p kinase activity in cells damaged in G_1 . Dun1p was immunoprecipitated from G_1 -synchronized wild-type cells (Y443) that were treated with (+; solid bars) or without (-; hatched bars) 80 J/m² UV and either maintained in G_1 or released from the G_1 block (G_1 -S) for 1 hr after irradiation. Bar graph shows the amount of radioactive incorporation into the peptide substrate, whereas the protein bands indicate the amount of immunoprecipitated Dun1p as determined by Western analysis. Net counts averaged over two independent experiments were calculated by measuring total counts incorporated and subtracting counts incorporated in reactions lacking peptide or control extracts lacking Dun1p. (D) *RNR3* induction in cells that were damaged at α -factor arrest and then blocked at the *cdc4* and *cdc7* arrest points. Northern blot analysis showing *RNR3* expression in *cdc4* (WS9110-3D), *cdc4rad9Δ* (WS9110-3D *rad9Δ*), *cdc7* (WS9120-10A) and *cdc4rad9Δ* (WS9120-10A *rad9Δ*) that were synchronized in G_1 with α -factor at 24°C, incubated at 37°C for 1 hr, treated with (+; solid bars) or without (-; hatched bars) 80 J/m² UV, released from α -factor and then maintained at 37°C for 1 hr after release and harvested for analysis. Bar graphs show relative levels of *RNR3* normalized to actin controls. At bottom, FACS profiles of cells from asynchronous populations, α factor-synchronized populations at the point of UV irradiation and following irradiation at the *cdc4* and *cdc7* arrest points where total RNA was isolated.

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 G_1 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* transcrip-

tion. 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 G_2 (60 min) and decreases as they enter mitosis. *rad9* cells are initially uninducible when irradiated in G_1 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 G_2 , however, *rad9* cells are once again uninducible relative to wild-type. Thus, it is mainly in G_1 or G_2 where *RAD9* appears to play a major role in trans-

Figure 3. Complementary roles for *POL2* and *RAD9* in *RNR3* induction at different stages of the cell cycle. Isogenic wild-type (Y300; solid bars), *rad9* (Y438; hatched bars), *pol2* (Y439; solid bars) and *pol2rad9* (Y440; hatched bars) were synchronized in G_1 with α -factor at 24°C, released from the block and irradiated with 80 J/m² UV at different times after release (shown in minutes). Bar graphs show relative levels of *RNR3* expression as determined by Northern blot analysis and normalized to actin controls. FACS analysis shows relative position of the cells at the point of irradiation (-UV, left) and 1 hr postirradiation at which point the cells were collected for Northern analysis [+UV (1h), right].

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 (*G*₁-S) and incubated for 1 hr prior to preparation of extracts. Lane 1 contains extract from $\Delta rad53$ 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.

ducing 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 *G*₁ 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 *G*₂ 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.

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/*G*₂ 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 *G*₁ 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 *G*₁. *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 *G*₂ at 36°C because of the generation of telomere-

Figure 5. *RNR3* induction in other checkpoint-defective mutant cells. (A) UV induction of *RNR3* in other members of the *RAD9* class of checkpoint mutants. Isogenic wild-type (TWY397), *rad24* (TWY399) and *mec3* (TWY316) asynchronous (Asy) or G_1 -arrested cells were UV-irradiated (solid bars; control, hatched bars), released from arrest (G_1 -S) when appropriate and RNA was harvested after 1.5 hr. Bar graphs show relative levels of *RNR3* expression for each sample. (B) UV induction of *RNR3* in other S-phase checkpoint-defective mutants. Samples were treated as in (A), *mec1* (Y306) and *rad53/sad1* (Y301) mutants compared with *Mec⁺* (Y300). (C) *RNR3* induction in wild-type (TWY397), *rad53/mec2* (TWY312), *mec1*(TWY308), *pol2* (Y439), *rad9* (TWY398), *mec3* (TWY316) and *rad24* (TWY399) that were incubated with 0.2 M hydroxyurea for 4 hr at 30°C (solid bars; control, hatched bars). *pol2* mutants were incubated at both 30°C and 37°C because it has been observed that their defects in checkpoint function are more severe at higher temperature (Navas and Elledge, unpubl.). (D) *cdc13*-mediated induction of *RNR3* is *RAD9* dependent. Northern blot analysis of total RNA isolated from wild-type (TWY397), *cdc13* (TWY431) and *cdc13rad9* double mutants (TWY72) using *RNR3* and actin as probes. Cells were synchronized in G_1 with α -factor at 24°C and then released from the α -factor block at 37°C. Cells were harvested at the indicated times and total RNA was prepared. The bottom panel shows the fold induction of *RNR3* normalized to actin and quantitated using a PhosphorImager.

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 G_2 , we assayed *RNR3* induction in wild-type, *cdc13*, and *cdc13rad9* cells that were synchronized in G_1 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 G_2 .

pol2rad9 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.

pol2rad9 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.

Figure 6. UV and HU sensitivity of *pol2rad9* mutants and suppression by RAD53. (A) UV sensitivity of wild-type (■), *pol2* (△), *rad9* (●), and *pol2rad9* (□) mutants at 28°C. Graph shows percent survival of cells plotted against increasing dose of UV. (B) HU sensitivity of *pol2rad9* compared with isogenic wild-type, *pol2* and *rad9* mutants. "+" indicates growth and "-" indicates lethality. At bottom, suppression of the HU sensitivity of *pol2rad9* (Y440) by RAD53. RAD53 expression is under the control of the *GAL1* promoter (pJA98). Yeast cells were streaked onto SC-uracil plates containing galactose supplemented with or without 10 mM HU. Suppression was scored by the ability to form colonies after incubation at 24°C.

Alternatively, *pol2* mutants in the presence of low amounts of HU might synthesize faulty DNA that activates the RAD9 checkpoint in G₂ allowing repair prior to mitotic entry. In *pol2rad9* mutants, cells would proceed into mitosis without fully repairing such lesions resulting in mitotic catastrophe. We cannot currently distinguish between these models. It should be noted that *mec1* mutants that completely eliminate signaling in this pathway are extremely HU- and UV-sensitive, much more so than *rad9* or *pol2* mutants alone. However, the HU and UV sensitivities of *pol2rad9* double mutants approaches that of *mec1* mutants, as does the degree of impairment of the UV-inducibility of *RNR3* (Figs. 2 and 5B).

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 (Lyall 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 in 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 G_1 , *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 G_2 based upon *cdc13*-mediated *RNR3* induction and the dependency for UV-induced cell-cycle arrest in G_2 . *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 G_1 and G_2 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 G_1 demonstrates that *RAD9* functions upstream of *RAD53*. The *cdc13*-mediated *RNR3* induction in G_2 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-

Table 1. Yeast strains used in this study

Strain	Genotype	Source
TWY397	<i>MATa ura3 his7 leu2 trp1</i>	Weinert et al. 1994
TWY398	<i>MATa rad9Δ::LEU2 his7 ura3 leu2 trp1</i>	Weinert et al. 1994
TWY308	<i>MATa mec1-1 ura3 trp1</i>	Weinert et al. 1994
TWY312	<i>MATa mec2-1 ura3 his7 trp1</i>	Weinert et al. 1994
TWY316	<i>MATa mec3-1 ura3 his3 trp1</i>	Weinert et al. 1994
TWY399	<i>MATa rad24-1 ura3 his7 leu2 trp1</i>	Weinert et al. 1994
TWY431	<i>MATa cdc13-1 ura3 his3</i>	Weinert et al. 1994
TWY 72	<i>MATa cdc13-1 rad9Δ::LEU2 his7 trp1 ura3 can1-100</i>	T. Weinert (University of Arizona, Tucson)
Y300	<i>MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Allen et al. 1994
Y321	Y300 + pUN70 (<i>URA3</i>)	Allen et al. 1994
Y301	<i>MATa sad1-1 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Allen et al. 1994
Y306	<i>MATa sad3-1 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Allen et al. 1994
WS9110-3D	<i>MATa cdc4-1 ade2-1 ura3-52</i>	Siede et al. 1994
WS9110-3D <i>rad9Δ</i>	<i>MATa cdc4-1 rad9Δ::URA3 ade2-1 ura3-52</i>	Siede et al. 1994
WS9120-10A	<i>MATa cdc7-1 ura3-52</i>	Siede et al. 1994
WS9120-10A <i>rad9Δ</i>	<i>MATa cdc7-1 rad9Δ::URA3 ura3-52</i>	Siede et al. 1994
Y438	<i>MATa rad9::HIS3 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	This study
Y439	<i>MATa can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pol2-12-TRP</i>	This study
Y440	<i>MATa rad9::HIS3 can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 pol2-12-TRP1</i>	This study
Y441	Y300 + pZZ74 (<i>GAP-HA DUN1 2μ URA3</i>)	This study
Y442	Y300 + pAB23BXN (<i>2μ URA3</i>)	This study
Y443	Y440 + pJA98 (<i>GAL-RAD53 URA3</i>)	This study
Y444	Y440 + pUN70 (<i>URA3</i>)	This study

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 ($OD_{600} = 0.4$) 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 YPD. After 1.5 hr at 24°C, aliquots were collected for flow cytometry (FACS) and the rest of the cells were quick-frozen 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 μ g/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 μ g/ml) for G₁ cultures, whereas G₁-S cultures were washed once with water and resuspended in YPD for 1 hr before harvesting and quick freezing in liquid nitrogen.

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 pAB23BXN (vector) were synchronized in G_1 with α -factor and treated with or without 80 J/m² UV-irradiation and either maintained in G_1 or released from the G_1 block (G_1 –S) for 1 hr after irradiation. Dun1 was immunoprecipitated from 200 μ g of yeast protein and peptide kinase assays were performed with the peptide LKKLTRRASFSQ 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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RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in *Saccharomyces cerevisiae*.

T A Navas, Y Sanchez and S J Elledge

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