Published in final edited form as:

DNA Repair (Amst). 2011 November 10; 10(11): 1086–1094. doi:10.1016/j.dnarep.2011.07.007.

The DNA damage checkpoint allows recombination between divergent DNA sequences in budding yeast

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Abstract

In the early steps of homologous recombination, single-stranded DNA (ssDNA) from a broken chromosome invades homologous sequence located in a sister or homolog donor. In genomes that contain numerous repetitive DNA elements or gene paralogs, recombination can potentially occur between non-allelic/divergent (homeologous) sequences that share sequence identity. Such recombination events can lead to lethal chromosomal deletions or rearrangements. However, homeologous recombination events can be suppressed through rejection mechanisms that involve recognition of DNA mismatches in heteroduplex DNA by mismatch repair factors, followed by active unwinding of the heteroduplex DNA by helicases. Because factors required for heteroduplex rejection are hypothesized to be targets and/or effectors of the DNA damage response (DDR), a cell cycle control mechanism that ensures timely and efficient repair, we tested whether the DDR, and more specifically, the *RAD9* gene, had a role in regulating rejection. We performed these studies using a DNA repair assay that measures repair by single-strand annealing (SSA) of a double-strand break (DSB) using homeologous DNA templates. We found that repair of homeologous DNA sequences, but not identical sequences, induced a *RAD9*-dependent cell cycle delay in the G2 stage of the cell cycle. Repair through a divergent DNA template occurred more frequently in *RAD9* compared to *rad9Δ* strains. However, repair in *rad9Δ* mutants could be restored to wild-type levels if a G2 delay was induced by nocodazole. These results suggest that cell cycle arrest induced by the Rad9-dependent DDR allows repair between divergent DNA sequences despite the potential for creating deleterious genome rearrangements, and illustrates the importance of additional cellular mechanisms that act to suppress recombination between divergent DNA sequences.

Keywords

heteroduplex rejection; DNA mismatch repair; DNA damage response; Rad9

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

The DNA damage response (DDR) plays a central role in ensuring that critical biological processes, such as immunoglobulin diversification, gamete development, and telomere homeostasis, occur with limited errors [1–3]. These processes rely on programmed genomic insults that are repaired in a highly regulated manner, and the DDR is essential for coordinating their repair with cell growth and division. Humans and mice with defects in the DDR exhibit increased genomic instability and can display increased incidence of cancer, neurodegeneration, immunodeficiency, or infertility (i.e. Ataxia telangiectasia, Nijmegen breakage syndrome, Down's syndrome, Alzheimer's disease [4]).

Genome stability is maintained by groups of proteins that recognize and repair DNA damage in the form of replication or recombination errors and chemically or radioactively-induced lesions [4–8]. DNA double-strand breaks (DSBs) are a cytotoxic type of DNA damage that can result from strand breakage associated with physical stress, ionizing radiation, endonuclease cleavage, stalled intermediates in DNA lesion processing, and replication fork collapse [9–16]. DSBs are often repaired by one of several forms of chromosomal recombination, and their timely and accurate repair is essential for avoiding the genomic rearrangements that can lead to disease.

In budding yeast the DDR is critical for promoting efficient repair of DSBs. Upon formation of a DSB, a single DNA strand is resected from each broken end in the 5' to 3' direction, exposing 3' single-stranded DNA (ssDNA). The ssDNA is immediately bound by RPA, followed by binding of complexes containing the Mec1/Tel1 PIKK protein kinases and Rad9 [17,18]. Rad9 is phosphorylated by Mec1/Tel1 and forms an oligomer which serves as a scaffold for Rad53, allowing for Rad53 autophosphorylation [19]. Rad53 is the central DDR transducer which signals to many downstream effectors to promote localization of DNA repair factors to sites of damage and delays cell cycle progression to ensure that the damage is repaired before cell division [19–21]. If a DSB fails to be repaired, the cell will either remain terminally arrested at the G2/M stage of the cell cycle or will undergo break adaptation and die after several divisions [22–24].

Though the DDR has been widely studied, our understanding of all of its downstream steps is far from complete. One area that is not well understood is the role of the DDR in the choice of DSB repair pathway and the recognition of the correct repair template for homologous recombination. DSB repair may occur by the non-conservative non-homologous end joining pathway (NHEJ), or by one form of conservative homologous recombination (HR) including classical double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA), or break-induced replication (BIR), all of which initiate with strand invasion or annealing of homologous DNA sequences which are then used as templates for DNA synthesis to fill in sequence gaps [25]. The current understanding is that the DSBR and SDSA pathways are preferred during the late S or G2 stage of the cell cycle or meiotic pachytene when chromosomes are in close proximity to a sister chromatid or homologous chromosome. In contrast, NHEJ is functional at all cell cycle stages, and therefore is primarily responsible for repairing breaks during the G0 or G1 stages in mammals (though it plays a smaller role in DSB repair in yeast) when sister chromatids are unavailable [26,27]. Finally, SSA and BIR are specialized for repair of DSBs within repetitive DNA elements and when only one DSB end has a homologous template, respectively [28].

Little is known about how the DDR affects the choice of homologous repair template during HR. Despite the fact that approximately 50% of the human genome is composed of repetitive DNA elements [29], homologous template choice for DSB repair is still chosen

with remarkable fidelity [30]. Still, a number of genome rearrangements between repetitive elements have been described that have been associated with cancers and neurological diseases, including familial breast and ovarian cancer as well as Charcot-Marie-Tooth disease [31–34], highlighting the need to further understand the mechanisms that control HR. Some of the factors required for homologous template choice have been identified [35–42]; however, the role of the DDR in this choice has not been explored. Here, we tested a role of the DDR in homologous partner choice using SSA between direct DNA repeats as a model. Previous work using this model [35] has shown that SSA between 205 bp repetitive elements spaced 2.6 kb apart is repaired efficiently by annealing of complementary DNA on resected ssDNA ends, cleavage of the 3' tails derived from the intervening non-homologous sequences, and filling of gaps by DNA synthesis and ligation to create a double-stranded DNA (dsDNA) deletion product (Fig. 1). However, SSA repair at the same locus is inefficient when the repetitive elements share less-than-perfect sequence identity, except when factors critical for disrupting the heteroduplex intermediate (Msh6 or Sgs1) are absent [35]. The process for disruption of divergent SSA intermediates, termed heteroduplex rejection, occurs by a conservative unwinding mechanism such that rejected intermediates still have the potential to repair correctly if the appropriate homologous template is available [36].

To determine whether the *RAD9*-dependent DDR is involved in the formation or rejection of heteroduplex SSA intermediates, we compared the effectiveness of heteroduplex rejection in a wild-type versus *rad9Δ* strain background. Unexpectedly, we found that heteroduplex rejection was less efficient in the presence of the DDR than in its absence; DDR allowed recombination between divergent sequences. Further analysis showed that a G2 delay occurred in wild-type strains that allowed divergent recombination, and inducing a synthetic G2 delay in *rad9Δ* mutants by adding nocodazole was able to restore the wild-type level of rejection. These results are the first to show a role for the DDR in allowing inappropriate error-prone DSB repair over error-free repair. This work also provides insights into how repetitive DNA can threaten the integrity of the genome and suggests a new explanation for why some disease-causing rearrangements could escape mechanisms that normally suppress them.

2. Materials and methods

2.1. Strains

Strains used in this study were identical to or derived from those used in Sugawara *et al.* [35] and Goldfarb and Alani [36]. These strains carry a duplication or triplication of the 5'–205 bp of the *URA3* gene (A) that is identical to or 3% divergent (F) from the wild-type sequence. The wild-type and parent strains were EAY1141 (A-A), EAY1143 (F-A), EAY1137 (A-A-A), and EAY1139 (A-F-A). Mutant derivatives were created by standard gene replacement using auxotrophic or drug resistance markers to create the following strains: EAY1392 (A-A *sgs1Δ::KANMX*), EAY1354 (F-A *sgs1Δ::KANMX*), EAY1387 (A-A *msh6Δ::KANMX*), EAY1388 (F-A *msh6Δ::KANMX*), EAY2375 (A-A *rad9Δ::KANMX*), EAY2376 (F-A *rad9Δ::KANMX*), EAY2566 (A-A *rad9Δ::TRP1 msh6Δ::KANMX*), EAY2567 (F-A *rad9Δ::TRP1 msh6Δ::KANMX*), EAY2404 and EAY2405 (A-A-A *rad9Δ::KANMX*), and EAY2551 and EAY2552 (A-F-A *rad9Δ::KANMX*).

2.2. Survival Assay

Strains were struck from –80°C freezer stocks and, after 2–3 days, single colonies were inoculated into 5 ml Yeast Peptone Dextrose (YPD) [43]. After growth at 30°C to saturation, cells were collected, washed in distilled water, and inoculated to a final dilution of 1:25 to 1:100 in 5 ml YP-lactate (2%). Lactate cultures were incubated at 30°C for 16–18 hours

until mid-log phase growth ($O.D._{600} = 0.4\text{--}0.6$), diluted to 1:2500 in distilled water, and 100 μ l was plated on YPD and YP-galactose (2%). Plates were incubated at 30°C for 2–3 days. SSA efficiency is presented as the ratio of colonies present on YP-galactose to YPD plates for each strain $\pm$ SD.

2.3. Southern Blot Analysis

Strains were struck from -80°C freezer stocks and, after 2–3 days, single colonies were inoculated into 5 ml YPD. After growth at 30°C to saturation, cells were collected, washed in distilled water, and inoculated to a final dilution of 1:100 or 1:200 in 250 ml YP-lactate (2%). Lactate cultures were incubated at 30°C for 16–18 hours until mid-log phase growth ($O.D._{600} = 0.4\text{--}0.6$). 40 ml of the culture was collected for the uninduced ($T=0$) time point and galactose (2%) was added to the remaining culture. Cultures were incubated for 5 hours, and 40 ml samples were collected at various points throughout the time course. Collected samples were pelleted and stored at -80°C for at least 16 hours, after which chromosomal DNA was isolated and subjected to Southern blot analysis as described in Goldfarb and Alani, 2005 [36]. The amount of SSA product at 5 hours after galactose induction was calculated as a fraction of starting material (uncut band intensity at $T=0$). In the indicated time courses, nocodazole (Sigma M1404) was added to a final concentration of 15 $\mu\text{g/ml}$ simultaneously with galactose. In these experiments, the overnight lactate cultures were incubated in the presence of 1% DMSO to facilitate the solubility of nocodazole in the cytosol.

2.4. Microscopy

Samples for assessing cell cycle stage by microscopy were collected simultaneously with Southern blot samples. 1 ml of culture was collected at each time point and fixed in 4% formaldehyde. Within 2 weeks of storage at 4°C, cells were suspended in VectaShield (Vector Laboratories, Burlingame CA), mounted to glass slides, and in some cases stained with DAPI to visualize the nucleus [44]. The slides were viewed by both light and fluorescence microscopy (Zeiss 38HE filter) at 100x using an oil immersion lens and were scored for bud size and location of the nucleus: No bud, randomly-placed nucleus (G1); small bud, randomly-placed nucleus (S); large bud, nucleus at the bud neck (G2); large bud, nucleus spanning the bud neck (M); large bud or pair of enlarged divided cells with asymmetric nuclear distribution (failed nuclear division). A small bud was defined as being less than half of the size of the mother, and a large bud was more than half the size of the mother. In cases when DAPI was not used, G2, M, and failed nuclear division were difficult to distinguish and were scored as: bud less than size of mother (G2/M) and bud same size as mother (failed nuclear division). In the latter case, both mother and bud were most often enlarged.

2.5. Fluorescence Activated Cell Sorting (FACS)

Samples for FACS were collected simultaneously with Southern blot and microscopy samples. 1 ml of culture was collected at each time point, washed in distilled water, and suspended in 70% ethanol to fix cells. After 1–3 days at 4°C, the ethanol was removed and prepared for flow cytometry as described in Lyndaker *et al.* 2008 [45]. Cells were processed immediately at the Cornell Flow Cytometry Core Laboratory.

3. Results

3.1. Divergent SSA recombination is decreased in the absence of the RAD9-dependent DNA damage response

We examined whether the DDR acts to suppress recombination between divergent sequences using a SSA assay [35,36]. In this assay, a reporter consisting of a duplication of the 5' – 205 bp of *URA3* followed by the recognition site for the HO endonuclease and 2.5 kb of λ DNA is positioned upstream of the *URA3* coding region (Fig. 1A). The HO endonuclease is expressed under the control of a galactose-inducible promoter and upon expression, will cleave the dsDNA between the *URA3* repeats and at no other location in the genome. The resulting break has the potential to be repaired by SSA, and has little probability of being repaired by NHEJ or any other HR pathway [35]. In one of the two isogenic strains, the 5'-*URA3* duplication is identical to the endogenous *URA3* sequence (A-A), and in the second strain (F-A), the duplication contains seven polymorphisms with respect to the endogenous sequence such that the level of identity between the two repeats is 97%. By creating a deletion or mutation of a gene of interest in these two strains and comparing the success of SSA, we can determine whether that gene has a role in rejecting heteroduplex recombination intermediates when the repeat sequences are divergent.

We assessed the efficiency of SSA in the A-A and F-A strains by both physical and genetic analyses (Materials and methods). We induced DSB formation by adding galactose to mid-log phase cultures and collected and plated cells at time intervals for up to 5 hours following induction. By 5 hours, break repair is complete and cells with repaired breaks begin to grow. We isolated genomic DNA at each time point, digested the SSA locus with *Bgl*II, and detected the presence of recombination intermediates and products by Southern blot using a dsDNA probe specific to a region downstream of the 5'-*URA3* repeats (Fig. 1A). Consistent with previous analyses [35,36], we saw that in a wild-type strain, SSA was about 90% efficient in repairing a DSB between identical repeats, but could only repair about 20% of DSBs between the divergent repeats (cells that fail to repair the HO induced DSB do not survive [22]), such that the ratio of identical repair to divergent repair (A-A/F-A) was 3.3 in cell viability assays (Table 1) and 4.8 in physical assays (Table 2). In contrast, strains lacking genes required for rejection (*msh6 Δ* or *sgs1 Δ*) displayed equivalent SSA efficiencies in both the A-A and F-A strains (and therefore an A-A/F-A ratio closer to 1.0), indicating that recombination between divergent repeats was no longer being suppressed (Tables 1, 2) [36].

To determine whether the DSB-induced DDR is important for homologous template choice, we compared the efficiency of heteroduplex rejection, as measured by the A-A/F-A ratio, in a *rad9 Δ* strain during SSA between the identical versus divergent repeats. We focused on *RAD9* because of its requirement for recognizing and responding specifically to DSBs and not to damage associated with replication stress [19]. Surprisingly, we found that loss of the DDR resulted in greater efficiency of heteroduplex rejection such that SSA repair between divergent repeats was allowed by *RAD9* (Fig. 1, Tables 1, 2). Though the absence of *RAD9* had no effect on the initial rate of SSA, it had a subtle, but significant, effect on repair at later time points (Fig. 1C). Furthermore, as shown in Table 1, survival in the presence of galactose was unchanged by *RAD9* when heteroduplex rejection was disabled by deletion of *MSH6*, indicating that the decreased survival of F-A *rad9 Δ* cells is due to the rejection of SSA between the divergent repeats.

Overall SSA product in *rad9 Δ* was decreased by 38% when the break occurred between identical repeats (A-A), but was reduced to greater extent (56%) when the break was between the divergent F-A repeats (Table 2), indicating that although the DDR promotes overall DSB repair, it permits divergent DSB repair to a greater extent than identical repair.

This apparent difference in the *rad9Δ* effect in A-A and F-A strains may reflect saturation to maximum repair in the A-A strain, rather than a homology-directed decision. Interestingly, SSA repair between A-A repeats as determined by Southern blot did not perfectly correlate with cell survival, suggesting that the absence of *RAD9* may allow an alternative repair pathway for breaks between identical repeats that produces a product not detectable by our methods.

3.2. *RAD9* does not affect homologous partner choice during SSA

To test whether *RAD9* has a direct role in the choice of an identical versus a divergent template for SSA repair, we utilized a variant of the SSA reporter in which an additional identical repeat is provided upstream of either the A-A or F-A locus [36]. In this situation, SSA can either occur between the proximal repeats creating a small deletion product or the distal repeats creating a large deletion product (Fig. 2A). Thus, cells that reject heteroduplex DNA between the F and A repeats can still form A-A duplexes, complete SSA, and survive galactose exposure. We determined the preference for formation of duplexes with proximal or distal repeats by evaluating the amount of small and large deletion products by Southern blot. Both wild-type and *rad9Δ* strains showed the same ~16-fold preference for forming homoduplexes with the distal repeat rather than forming heteroduplexes with the proximal repeat, and little preference for either template when both repeats were identical [35,36]. This result indicates that *RAD9* does not play a role in template preference (homologous versus divergent).

3.3. Heteroduplex rejection elicits a *RAD9*-dependent cell cycle delay

rad9Δ strains exposed to radiation-induced DSBs are inviable and do not show a cell cycle delay at the large-budded G2 stage of the cell cycle [46]. Weinert and Hartwell showed that the cell cycle delay induced by the DDR is important to allow repair of radiation-induced DSBs. Consistent with this and the observation that a single DSB within the genome can elicit a DDR [22,23], we found that induction of the HO-catalyzed DSB between the 5'-*URA3* repeats caused a G2 delay that was *RAD9*-dependent (Fig. 3A *left*, Table 3). The G2 delay was maintained indefinitely when the repeats were divergent, similarly to the arrest observed in the presence of a persistent DSB [22]; however, due to the delayed accumulation of anucleate cells ("failed nuclear division" phenotype observed by microscopy, data not shown) it appeared that at least some cells completed the first round of cell division and encountered chromosome segregation problems in subsequent divisions. G2-stage haploid cells are those defined as having 2n DNA content, after DNA replication but before mitosis. Fluorescence-activated cell sorting (FACS) confirmed that the delay at the large-budded stage was due to a delay of mitosis following replication, since a similar *RAD9*-dependent delay occurred at the 2n stage (Fig. 3B, *left*).

3.4. Nocodazole-induced cell cycle delay in *rad9Δ* mutants rescues divergent SSA recombination

To determine whether the absence of the G2 delay alone contributes to the enhancement of heteroduplex rejection, we attempted a synthetic rescue of the G2 delay in the *rad9Δ* strains. Microtubule de-stabilizing drugs have traditionally been used to reconstitute G2 delays in yeast strains lacking DNA damage responses [46]. Since the *RAD9*-dependent G2 delay appeared to initiate shortly after galactose induction and to reach a maximum by approximately 3 to 4 hours after induction, we reasoned that by adding an appropriate concentration of the microtubule destabilizer nocodazole simultaneously with galactose, we could induce a G2 delay that initiated and completed in a similar time frame to the delay normally imposed by *RAD9*. We repeated the SSA assays as usual, except that 15 μg/ml nocodazole was added at T=0. Analogously to the Weinert and Hartwell study [46] where the drug MBC protected *rad9Δ* mutants from ionizing radiation damage, nocodazole

treatment enhanced the G2 delay in both wild-type and *rad9Δ* strains, regardless of the identity of the upstream 5'-*URA3* repeat (Fig. 3A and 3B, *right*, Table 3) and increased the fraction of DSBs repaired in all strains. The nocodazole-induced enhancement in repair in *rad9Δ* strains was larger for DSBs between divergent repeats (1.9-fold) than for DSBs between identical repeats (1.4-fold; Table 2). In the wild-type strains, nocodazole produced only a small enhancement in F-A repair (1.4-fold; Table 2), but had no effect on repair between A-A repeats. This difference adjusts the A-A/F-A ratio for *rad9Δ* to a ratio like that of wild-type in the absence of nocodazole (4.8; Table 2), while the rejection efficiency of wild-type in the presence of nocodazole was reduced even further (3.3; Table 2). These results indicate that the *RAD9*-dependent G2 delay alone is sufficient to allow SSA between the F-A repeats, and thus appears to allow greater opportunity for heteroduplex intermediates to escape rejection.

4. Discussion

DSBs are thought to be some of the most cytotoxic forms of DNA damage. The presence of even a single DSB within the genome induces a DNA damage response that can cause lethality if the DSB is difficult or impossible to repair [22]. However, improper repair can result in genome rearrangements and the potential for unequal nuclear division and chromosome loss [47]. How does the cell determine the ideal repair pathway for DSB repair? For the most part, this choice depends on the cell cycle stage during which the DSB forms. For example, in mammals repair by HR occurs during late S and G2 because homologous templates are available and in close proximity to the break site, whereas during other stages of the cell cycle NHEJ takes on the primary role of DSB repair [27].

It is thought that all of the different modes of DSB repair work in both collaboration and competition with each other to ensure that DSBs are not left unrepaired, and preference decisions are coordinated with cell cycle control. For example, the decision between NHEJ and HR is modulated by control of DSB end resection by cyclin-dependent kinases (CDKs) [27,48]. Rad9 localization to DSB ends is thought to serve as a physical block to resection, requiring phosphorylation by S- and G2-stage expressed CDKs to be removed [49]. Rad9 has also been shown to be important for channeling mitotic HR to repair with the sister chromatid; *rad9* mutant yeast display increased chromosomal translocations [50].

Following the decision to repair a DSB by HR, the cell must then distinguish the correct homologous template for repair synthesis. This decision is made in part by control of Rad51 association to resected DSB ends. Loss of *RAD51* in yeast results in increased SSA-mediated translocations and it appears that Rad51 displacement from resected ends directs the choice to repair by SSA [51]. Consistent with this, Rad51 overexpression leads to enhanced gene conversion and genome instability in mice, illustrating the need for the proper balance between repair pathways [52]. The second line of defense against incorrect homolog choice is disruption of heteroduplex repair intermediates between non-identical sequences. Heteroduplex rejection requires the concerted action of mismatch recognition proteins, helicases, endonucleases, and topoisomerases [35–37]. Whether the DDR modulates localization or activity of Rad51 and heteroduplex rejection factors remains to be determined.

Here, we show that loss of the *RAD9*-dependent DDR enhances the ability of the cell to suppress SSA between divergent repeats (Fig. 1, Tables 1, 2). The absence of *RAD9* did not affect the rate of SSA (ruling out the affect that *RAD9* loss may have on blocking end resection), nor did it play a direct role in a homology-directed decision (Fig. 2B,C). Treating a *rad9Δ* strain with a nocodazole-induced cell cycle delay allowed SSA to a greater extent in

F-A strains than A-A, shifting heteroduplex rejection (A-A/F-A) in *rad9Δ* mutants closer to that seen in wild-type strains (Table 2).

Our results show that the *RAD9*-dependent DDR can permit recombination between nonallelic sequences due to its ability to delay the cell cycle in G2 when HR repair is prominent. Observations in this paper and elsewhere [51] suggest that as long as normally-encountered levels or easy-to-repair types of DNA damage occur, the DDR can respond effectively and promote the most conservative form of repair possible in its present cell cycle stage. However, when higher-than-normal levels or difficult-to-repair damages are encountered, the conservative repair pathways become “overwhelmed,” and instead of simply eliminating the damaged cells, the DDR permits cell survival via use of non-conservative repair mechanisms. For example, Argueso *et al.* [53] showed that acute levels of DNA damage in yeast result in an abundance of chromosomal translocations between unlinked repeats, and that SSA is primarily responsible for these translocations.

This strategy of promoting cell survival over the potential for mutagenesis is similar to the induction of translesion synthesis (TLS) observed when replication forks are damaged or stalled. Organisms ranging from bacteria to humans possess a collection of specialized error-prone DNA polymerases that step in when the high-fidelity polymerases encounter lesions that block their paths [54]. Although TLS promotes cell survival during replication stress it also enhances the potential for base substitutions, microsatellite instability, and even gross chromosomal rearrangements [55–57]. We have shown in this study that the DDR surprisingly permits error-prone recombination, and it is known that the DDR promotes TLS. For example, DDR induction by stalled replication forks in *S. pombe* results in the up-regulation of the errorprone DinB polymerase and its recruitment to chromatin by the Rad9-Rad1-Hus1 (9-1-1) checkpoint complex [58,59]. In addition, mammalian polymerase η is dependent upon ATR for its role in recovery from UV irradiation, and the budding yeast S-phase checkpoint is responsible for suppressing TLS during exposure to methylmethane sulfonate [60,61]. However, the difference between these examples and the role of the DDR in rejection described here is that they occur through an active mechanism, i.e. a direct signaling event, rather than a passive effect of cell cycle delay. Although our study has not uncovered a more direct control of heteroduplex rejection by the DDR, we cannot rule out the possibility that rejection is controlled by checkpoint factors upstream of Rad9. In support of this, both Msh6 and Sgs1 are likely to be controlled by Mec1 rather than Rad9 since Msh6 was shown to be phosphorylated at two sites by Mec1 [62], and Sgs1 has several S/TQ motifs which are commonly targets of Mec1 [63]. Due to the requirement for Mec1 in SSA [64], we were unable to test rejection in a *mec1* strain using the SSA assay; however, studies with *msh6*-phosphorylation mutants are planned.

Approximately 50% of the human genome is made of repetitive DNA, much more than in the yeast genome [29], and it is estimated that the average human cell must repair about ten DSBs per day [65]. Disease-associated rearrangements between endogenous repeats have been described, but are not as common as these observations and work in yeast by Argueso *et al.* [53] would suggest. This lack of rearrangements between repeat sequences could be explained by the more frequent use of NHEJ in mammalian cells. Provided there is only limited damage, NHEJ could promote repair with genomic modifications confined to the region of the DSB, instead of creating the potential for translocations that would result from non-allelic homologous recombination.

Perhaps the relatively low incidence of rearrangements between repetitive sequences in eukaryotes is better explained by their residence in tightly packed heterochromatin domains, which might limit their exposure to DNA-damaging agents as well as recombination factors [66–69]. Recent work by Chiolo *et al.* suggests that repeat recombination is prevented in

heterochromatin because DSBs are recognized and processed for HR very rapidly, and are protected from Rad51 binding until the breaks are re-localized, in a checkpoint kinase-dependent manner, to a “safe,” presumably repeat-free location at the heterochromatin periphery [70]. Earlier studies suggest that DSBs formed within heterochromatin are repaired more slowly than breaks within euchromatin, and heterochromatin may additionally be protected from DSB formation in other ways such as more effective scavenging of reactive oxygen species [71–73].

Even though the HO-induced DSB used in our SSA assay is not expected to reside within heterochromatin, unrepairable breaks in yeast (i.e. like those in the F-A construct) re-localize to the nuclear periphery in a checkpoint-dependent manner [74,75]. The presumed euchromatic nature of our system also may explain the lack of DDR regulation of heteroduplex rejection; checkpoint regulation of HR fidelity and partner choice may be more prominent in heterochromatic regions, as suggested by studies in human cells in which ATM signaling became increasingly important for DSB repair with increasing chromatin complexity [73], or may be specifically upregulated during late S-phase, when heterochromatic repeats become exposed during replication. In addition to the above strategies, certain cell types in higher organisms may have evolved other forms of checkpoint regulation to avoid non-allelic recombination, such as minimizing time spent in G2-phase, down-regulating the DNA damage response, requiring a certain level of damage for full activation of the DNA damage response, or inducing apoptosis when persistent breaks or extensive damage is encountered [48,76–78].

Despite the many lines of defense against aberrant recombination in repetitive DNA, mammalian cells still may not be equipped to handle situations of acute DNA damage, such as overexposure to radiation or chemical mutagens, or when conservative repair pathways are compromised, as in pre-cancerous or tumor cells. This would allow some non-allelic recombination to slip through, and either cause disease or add more complexity to an already existing cancer.

Highlights

- Repair of divergent DNA sequences induced a DNA damage response cell cycle delay.
- Cell cycle arrest allows repair between divergent DNA sequences.
- Cellular mechanisms suppress recombination between divergent DNA sequences.

Abbreviations

DDR	DNA damage response
SSA	single-strand annealing
DSB	double-strand break
BIR	break-induced replication
HR	homologous recombination
ssDNA	single-stranded DNA
NHEJ	non-homologous end joining
SDSA	synthesis-dependent strand annealing

TLS translesion synthesis

Acknowledgments

We are grateful to the Alani lab and Bob Weiss, Marcus Smolka, Ted Weinert, Marco Foiani, and Max Gottesman for fruitful discussions. This work was funded by NIH GM53085 and NIH training grant support for C.M.G. awarded to Cornell University.

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Fig. 1. Heteroduplex rejection is enhanced in a *rad9Δ* strain

(A) The SSA assay. Two strains, A-A and F-A, possess a partial duplication of *URA3*, an HO cut site, and 2.5 kb of λ DNA upstream of the endogenous *URA3* locus. Induction of HO endonuclease expression produces a unique DSB between the repeats that is repaired efficiently by SSA in A-A, but inefficiently in F-A due to the rejection of the heteroduplex intermediate created by annealing of the divergent *URA3* repeats (3% divergence). SSA repair can be quantified by Southern blot using a probe downstream of the duplicated region of *URA3* to detect uncut (8.3 kb), cut (4.8 kb), and product (5.5 kb) species following HO expression. Successful SSA will promote cell survival, but cells that try to repair by SSA (perhaps multiple times) but ultimately fail will suffer the lethality caused by a persistent

DSB. (B) Southern blot analysis of HO-induced DSB formation and repair at the indicated times following induction of HO expression by addition of galactose. Representative blots of 3–6 independent experiments are shown. (C) Quantification of the data presented in B, normalized to the loading control and plotted as the fraction of starting material that is repaired as SSA product (Uncut at T=0/Product at T=5). Product at T=0 was set to 0 and subtracted from product at each time point.

Fig. 2. *RAD9* does not play a role in homologous vs. homeologous template choice

(A) A variation on the SSA assay in Fig. 1A with an additional partial duplication of *URA3* 2.9 kb upstream of the first duplication. After strand resection (I), the duplex intermediate can form between the endogenous *URA3* sequence and either the distal (IIa.) or proximal (IIb.) repeat. In the former case, a large deletion will result (IIIa.) and small deletion will result from the latter case (IIIb.) Both products can be detected and quantified by Southern blot and distinguished by their mobility on the gel (2.9 vs. 5.5 kb). (B) Southern blot detection of HO-induced DSB formation and repair by SSA as large deletion and small deletion products for wild-type and *rad9* Δ when all repeats are identical (A-A-A), or when the proximal repeat is 3% divergent (A-F-A). Representative blots are shown. (C) Southern

blot quantification of small and large deletion products at 5 hours after galactose addition expressed as a fraction of total product (small + large). Averages of 3–4 independent experiments are shown along with the average small deletion/large deletion ratio for each strain $\pm$ SD.

Fig. 3. G2 delay alone is sufficient to permit homeologous recombination

(A) Cells were fixed in 4% formaldehyde at the indicated times following induction of HO expression by galactose addition in the absence (*left*) or presence (*right*) of 15 μ g/ml nocodazole (added simultaneously with galactose). The percentage of G2/M stage cells was determined by counting large-budded cells under light microscopy for wild-type and *rad9Δ* strains. For the left panels, the averages of three independent experiments ($\pm$ S.D.) are shown. For the right panels, a representative of two independent experiments is shown (see also Table 3). (B) Cells were fixed in 70% ethanol at the indicated times following induction of HO expression by galactose addition in the absence (*left*) or presence (*right*) of 15 μ g/ml

nocodazole (added simultaneously with galactose). The percentage of G2/M stage cells was determined by flow cytometry. A representative of two independent experiments is shown.

Table 1

SSA repair efficiency* as determined in survival assays

Relevant genotype	A-A	F-A	A-A/F-A
wild-type	0.78 ± 0.14	0.23 ± 0.07	3.3
<i>msh6Δ</i>	0.76 ± 0.09	0.51 ± 0.01	1.5
<i>rad9Δ</i>	0.81 ± 0.11	0.13 ± 0.02**	6.4
<i>rad9Δ msh6Δ</i>	0.70 ± 0.12	0.53 ± 0.13	1.3

* Survival of indicated strains expressed as colony forming units on galactose/glucose. Mean survival ± SD for 3–12 experiments is shown.

** Significantly different from wild-type (Wilcoxon-Mann Whitney test, $p < 0.001$).

Table 2

SSA repair efficiency* as determined by Southern blot analysis

Relevant genotype	A-A	F-A	A-A/F-A
wild-type	0.86 ± 0.14	0.18 ± 0.04	4.8
<i>msh6Δ</i> **	1.12 ± 0.26	0.86 ± 0.07	1.3
<i>rad9Δ</i>	0.53 ± 0.09 ***	0.08 ± 0.04 ***	6.6
wild-type + nocodazole	0.82 ± 0.13	0.25 ± 0.04	3.3
<i>rad9Δ</i> + nocodazole	0.72 ± 0.11	0.15 ± 0.01	4.8

* SSA product at T=5 hr following galactose induction relative to uncut at T=0 hr expressed as the mean ± SD for 3–11 experiments. Nocodazole was included in time courses as described in the Materials and methods.

** The *msh6Δ* data were previously reported in Goldfarb and Alani [36].

*** Significantly different from wild-type (Wilcoxon-Mann Whitney test; p = 0.009 for A-A, p = 0.03 for F-A).

Table 3

Percentage of wild-type and *rad9Δ* cells in G2/M following HO induction.

Genotype	Hrs post induction	Large budded cells (%)			
		- nocodazole		15 μg/ml nocodazole	
		A-A	F-A	A-A	F-A
wild-type	0	27 ± 8	27 ± 8	28 ± 11	31 ± 3
wild-type	5	45 ± 10	93 ± 7	84 ± 4	85 ± 8
<i>rad9Δ</i>	0	37 ± 10	25 ± 8	28 ± 3	28 ± 2
<i>rad9Δ</i>	5	44 ± 8	55 ± 10	85 ± 5	88 ± 2

Wild-type and *rad9Δ* A-A and F-A strains were fixed in 4% formaldehyde at 5 hrs following induction of HO expression by galactose addition in the absence or presence of 15 μg/ml nocodazole (added simultaneously with galactose). The percentage of G2/M stage cells (n = 3 ± S.D., at least 100 cells counted per time point) was determined by counting large-budded cells under a light microscope.