Review

The Two Faces of *Saccharomyces cerevisiae* RAD9 Function in Homologous Recombination: Suppressor and Promoter of Genome Instability

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Review

The Two Faces of *Saccharomyces cerevisiae* RAD9 Function in Homologous Recombination: Suppressor and Promoter of Genome Instability

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Abstract

Recombinogenic DNA damage can initiate chromosomal rearrangements that can alter gene expression or accelerate cancer progression in higher eukaryotes. Thus, there is a critical need to identify genes that suppress chromosomal rearrangements and environmental exposures that promote genetic instability. Cell cycle checkpoints modulate the cell cycle so that DNA repair occurs before the replication or segregation of damaged chromosomes. *Saccharomyces cerevisiae* (budding yeast) *RAD9* was the first cell cycle checkpoint gene identified, which initiated intensive research studies into the mechanisms of checkpoint activation and the phenotypes of checkpoint mutants. The budding yeast *Rad9* protein serves as both an adaptor and scaffold that facilitates downstream effector activation to orchestrate a DNA damage response at multiple stages of the cell cycle, which facilitate double-strand break (DSB) repair by sister chromatid recombination. However, the role of *RAD9* in homologous recombination and in suppressing gross chromosomal rearrangements (GCRs) is not completely understood. In this review we discuss how *RAD9* can promote genome instability resulting from aberrant DNA replication intermediates, while suppressing DSB-associated rearrangements. We also discuss possible mechanisms accounting for the synergistic increase in genomic instability in double mutants defective in both *RAD9* and recombinational repair. We emphasize that while there is an overlap between checkpoint and recombinational repair pathways, *RAD9* and checkpoint pathways can function independently to suppress chromosomal instability. These studies thus elucidate checkpoint mechanisms that control homologous recombination between repeated sequences.

Keywords: homologous recombination; Genome instability; DNA damage; cell-cycle checkpoint; budding yeast

1. Introduction

Biological organisms have finely tuned DNA repair pathways to maintain genetic integrity. Such mechanisms repair a multitude of DNA lesions, including base pair damage, bulky DNA adducts, DNA cross-links and single and double-strand breaks (DSBs). However, cell cycle progression in the presence of unrepaired DNA lesions, can lead to replication fork collapse and the persistence of chromosomal fragments and genotoxic lesions, which generate mutations and chromosomal rearrangements in subsequent cell cycles. Hartwell and Weinert [1] coined cell cycle checkpoints as "control mechanisms that enforce dependency in the cell cycle". *RAD9* [2] was the first identified cell-cycle checkpoint gene. Cell-cycle checkpoint genes arrest or delay the cell cycle so that repair of DNA is completed before DNA lesions are replicated or segregated to the next cell cycle. This is particularly important in DSB repair, where aberrant repair of DSBs can reshape the genome via recombination between repeated sequences [3], while the persistence of a single DSB can confer lethality [4]

Cell cycle checkpoints include those that function at G_1/S, intra S phase and at G_2/M. Components of these checkpoints are well-conserved from yeast to mammalian cells. In general,

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protein sensors recognize DNA damage, which, in turn, activate apical kinases that signal to effector kinases, amplifying the DNA damage signal (for review, see [5,6]). These kinases, in turn, activate factors that arrest the cell cycle, modulate DNA repair, and control the transcriptional response to DNA damage [7,8]. While both apical and effector kinases can directly and indirectly promote their own self-regulation (for review, see [6,9]), phosphatases can directly remove phosphates from activated targets, and once DNA repair is completed, the absence of the DNA damage switches off activation signal (for review see, [10]).

Checkpoint activation is initiated by diverse DNA structures. While single-stranded DNA (ssDNA) is a general signal for checkpoint activation in *Saccharomyces cerevisiae* (budding yeast), DNA damage signals include DSBs [10–12], stalled replication forks [13], unresolved Holliday structures [14], ssDNA at telomeres [15,16], fragile DNA sites [17]. and unresolved DNA replication intermediates, such as 5′ single-strand flaps [18], trapped topoisomerase-DNA structures [19,20]. However, developmentally programmed DSBs, such and those generated by Spol11 in meiosis [21] and programmed HO endonuclease-induced breaks during mating-type switching, do not generate a checkpoint response [22].These observations indicate that activation of the checkpoint response is tightly controlled and partially dependent on the efficiency of repairing DNA lesions, such as DNA DSBs.

The redundancy of DSB-mediated repair pathways in eukaryotic organisms confers resistance to ionizing radiation and radiomimetic chemicals. These pathways include non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homologous recombination (HR) (for review, see [23]). HR pathways for DSB repair (Figure 1) include gap repair mechanisms, single-strand annealing (SSA), and break-induced replication (BIR) [23]. Common steps in HR pathways are the resection of the DSBs to reveal ssDNA, formation of a recombination intermediate involving heteroduplex DNA, and resolution of the heteroduplex [23]. The choice of which pathway is used in DSB repair depends on the cell cycle phase and ploidy; for example, NHEJ facilitates DSB repair in G1 haploid cells since sister chromatids and homologs are absent [4]. Considering the abundance of repeated sequences in yeast, DSBs could initiate diverse genome rearrangements [3], including deletions, duplications, and translocations (Figure 2).

The higher radiation resistance of G_2 diploid cells, compared to G_1 diploid cells [24,25] and the significant role of HR in DSB repair, supports the assertion that sister chromatids are the preferred substrates for recombinational repair [25]. Considering that sister chromatids are transient in the cell cycle, checkpoint control of DSB repair by has been intensively studied [26]. The central players in this pathway in budding yeast are the PI-3K like kinase encoding genes *TEL1* and *MEC1* [27], which are the orthologs of the ataxia telangiectasia mutated (ATM) and ATM-related gene (ATR), respectively [28]. DSB signaling involves the rapid recruitment of the Mre11/Sae2 complex and subsequent resection, which leads to ssDNA bound to single-strand binding protein (RPA) (Figure 3). Sae2 activation, in turn, is controlled by the Cdk2 so that resection occurs at the G_2 stage of the cell cycle [29]. The ssDNA is then a substrate for binding by the Mec1-Ddc2 complex, which is facilitated by the 9-1-1 complex. Mec1 also phosphorylates DNA repair proteins, including Sgs1 [30], Rad51 [31], Rad55 [32], and Exo1[30] that facilitate sister chromatid recombination. The Mec1 kinase phosphorylates Rad53 [33], the CHK2 ortholog, and Chk1 [34]. Rad9 serves to bind Rad53 and facilitates Mec1's phosphorylation of Rad53, which then triggers Rad53 autophosphorylaton and its release from Rad9 [35,36]. Rad53, in turn, phosphorylates effector proteins, whose functions are to inhibit late replication firing [13] , upregulate deoxynucleotide levels [37] and facilitate formation of Rad51 filaments [32]. Cell cycle arrest is achieved by Mec1-dependent activation of Chk1, which in turn phosphorylates Pds1 (securin), which blocks the degradation of cohesin by anaphase promoting complex (APC^{Cdc20}) [38]. Rad53 (Chk2) inhibits Cdc20-Pds1 interaction [39] and phosphorylates Dun1, which in turn, inhibits mitotic exit by activating the Bfa1-Bub2 complex [40]. Additional roles of the checkpoint response are to inhibit de novo telomere addition [41], promote the mobility of broken chromatids [42,43], and inhibit asymmetric resection [44], which could promote genome rearrangements [45]. Thus, checkpoints serve to both arrest the cell cycle and facilitate DNA repair.

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An intermediate substrate in both checkpoint signaling and recombinational repair is ssDNA bound to single-strand binding protein [46,47]. ssDNA generated by DSB resection is a substrate for Rad51 filament formation, which is facilitated by Rad52, Rad55, and Rad57 (for review see [48]). Rad51-coated DNA then catalyzes DNA strand invasion and with the assistance of Rad54 generate Holliday intermediates. The Sgs1 helicase can abort recombination intermediates [49], while additional proteins, such as Yen1, can resolve recombination intermediates to yield crossover events [50]. DSB processing thus produces intermediates which could bind either MR repair proteins or checkpoint proteins. Rad9 controls resection through its interaction with the Mrell complex [51], thus affecting both DSB-repair mechanisms and checkpoint signaling.

While the checkpoint response and RAD50 group genes are required for efficient DSB gap repair and ionizing radiation resistance, individual genes within the RAD50 group are not required at all stages of DNA damage-associated or spontaneous HR. Differential requirements can be due to multiple sources of spontaneous DNA lesions, including DNA replication errors, transcription, and base excision repair. While all HR events require *RAD52* [48], *RAD51* mutations only modestly affect spontaneous SCR [52] and spontaneous unequal sister chromatid recombination events (uSCR) are *RAD51*-independent [53]; additional pathways have been identified that participate in uSCR [54]. On the other hand, heteroallelic recombination is *RAD51*-dependent [48]. Rad50-mediated resection delays mating-type switching [55] but is not required to initiate X ray-associated crossovers [56]. Other studies have also shown multiple pathways for both SSA and BIR events [57,58]. *RAD9* functionality at all stages of the cell cycle [59] thus underscores the importance of understanding checkpoint function in diverse HR events and a better understanding of the Rad9 protein.

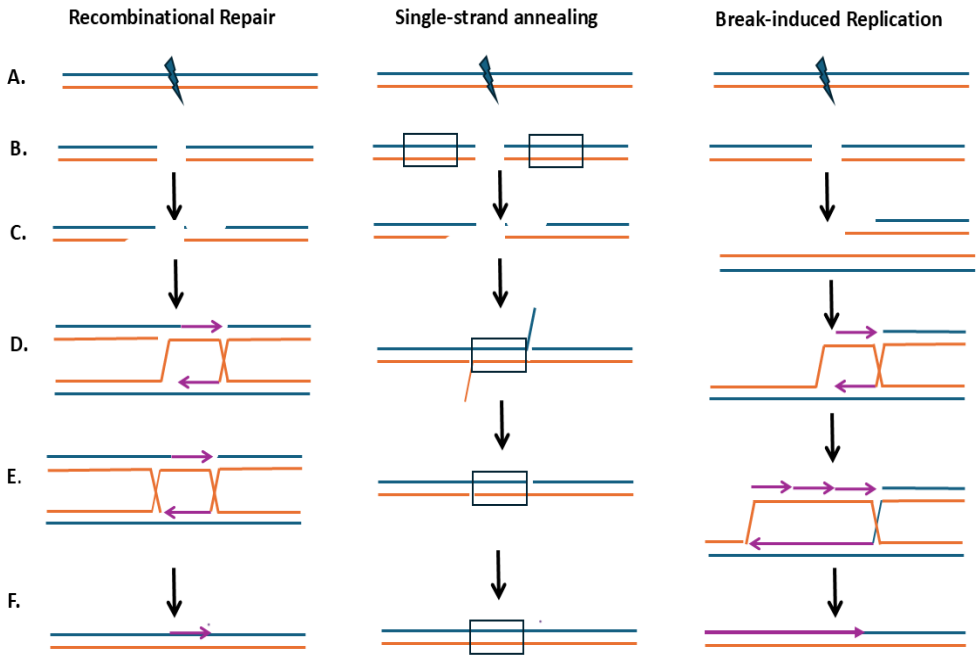

Figure 1. Double-strand break (DSB) repair pathways including sister chromatid recombinational repair (left panel), single-strand annealing (middle panel), and break-induced replication (right panel). A). The initiation of the DNA lesion, B). The position of the break, C). The resection of the break revealing 3′ overhangs, D). Homology search and annealing complementary DNA. E). DNA single-strand gaps that are filled by DNA polymerases where newly synthesized strands are shown in purple, F). Resolution, ligation of nicks, and reconstitution of the chromatid. Single strands of the 5′ -3′ polarity are shown in blue and 3′-5′ polarity is shown in yellow. The newly synthesized DNA is shown in purple, where the arrow indicates the 3′ polarity.

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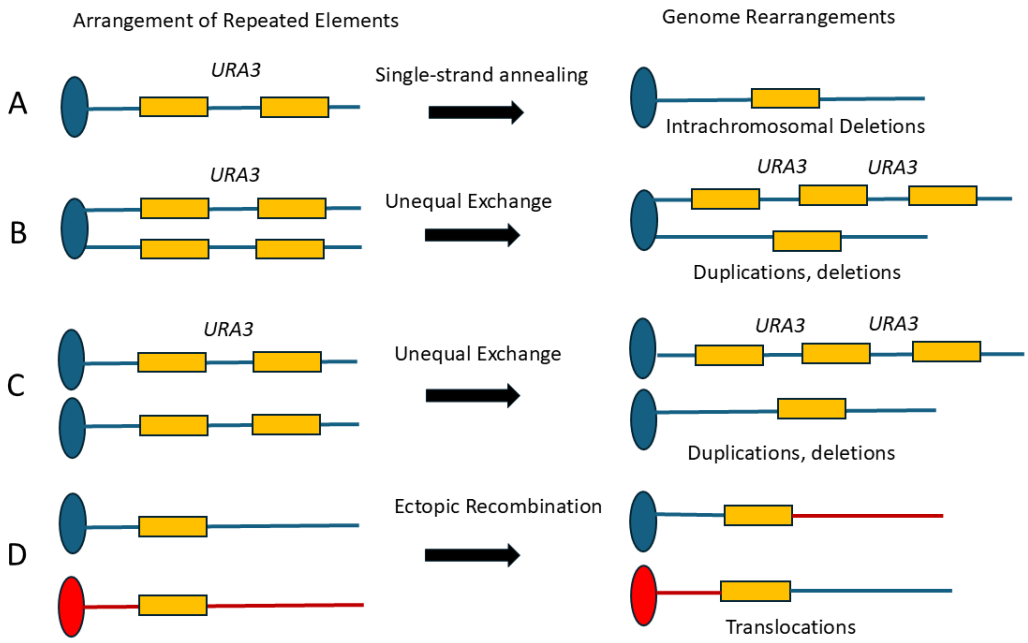

Figure 2. Arrangements of repeated sequences aligned in direct orientations on (A) chromosome, (B) sister chromatids, (C) homologs, and (D) non-homologous chromosomes. Recombination between these repeats generates, deletion, duplication, and reciprocal recombination. The chromosome is shown as a single line representing duplex DNA. For simplicity, the left arms of the chromosomes are not shown. The oval represents the centromere, and the rectangular box represents a repeated sequence. The blue and red colors are indicative of two different non-homologous chromosomes. The *URA3* is shown as an example of a gene located between repeated sequences.

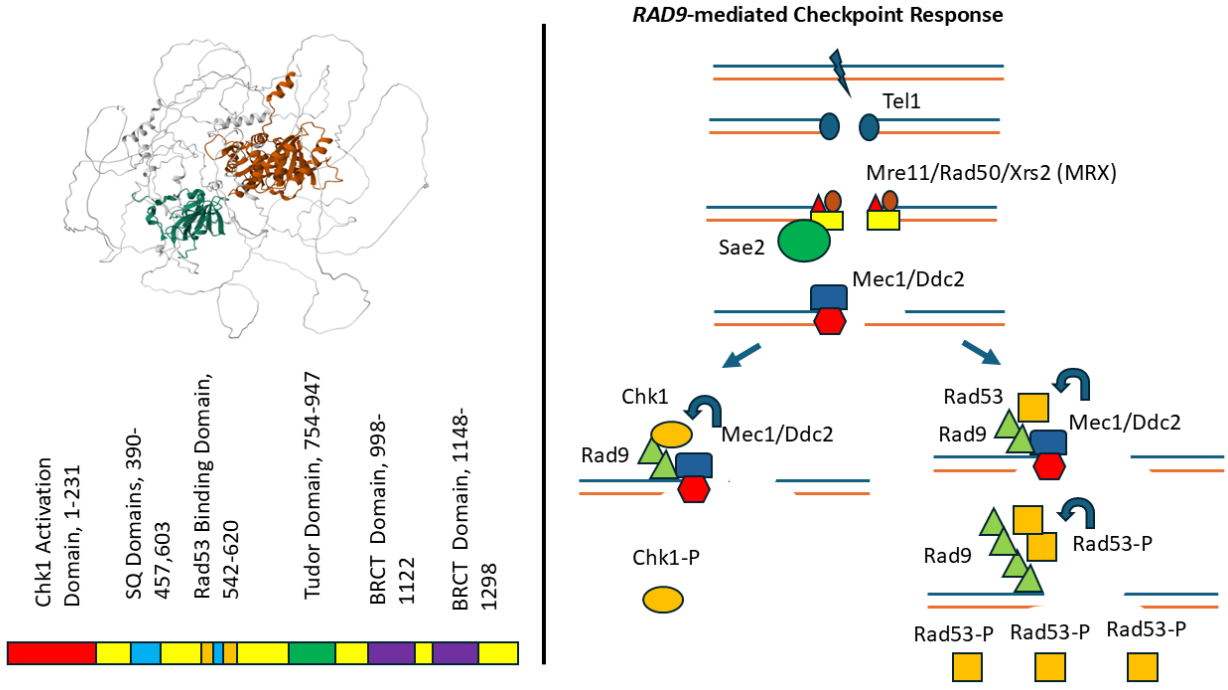

Figure 3. Domain structures and alpha-fold predicted structure of the Rad9 protein (1309 amino acids) and Rad9-mediated checkpoint pathway. On the left are the domains within the amino acid sequence, starting from the N-terminal to the C-terminal end. Each color code represents a different domain; red, blue, orange, green and purple represent the Chk1-activating domain, the SQ domain, the Rad53 binding domain, the tudor domain, the

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BRCT domain, respectively. The alpha fold structure is shown on the right, where the highest confidence structures are colored green and dark brown; these two domains cover both the tudor and the BRCT domains, respectively. On the right is the Rad9-mediated checkpoint pathway. The checkpoint pathway is initiated by a double-strand break (DSB), followed by Tel1 binding, MRX recruitment of Sae2 and nucleases, and Mec1 Ddc2 recruitment. Rad9 protein concentrates at the restricted DSB by binding to modified chromatin and then serves as a scaffold to recruit Chk1 (left) and Rad53 (right). Mec1 phosphorylates Chk1 and Rad53; Rad53p catalyzes its own phosphorylation and the polyphosphorylated Rad53 is released from the Rad9 scaffold.

2. Rad9 Protein and Function

The yeast *RAD9* gene encodes a protein of 1309 amino acids and is an ortholog of the human 53BP1 and the Schizosaccharomyces pombe *crb2*$^{+}$ genes [60,61], and shows similarity the human BRCA1 gene [61]. *RAD9* mediates DNA damage-activated checkpoints at G1 [63]), S [18,64], and G_2/M stages of the cell cycle [65]. An artificial intelligence (AI)-generated alpha fold structure [59] of the Rad9 protein is shown in Figure 3. This structure indicates both predicted and disordered domains; namely, with high confidence shown for the predicted structures of the tudor domains. The relevant structure includes five major features; these include the Chk1-activating domain (CAD), the Mec1/Tel1-phosphorylated SQ/TQ cluster domain (SCD), the Rad53 binding domain, the hydrophobic tandem tudor domain, and the BRCT domain [61]. Regulation is achieved by 98 phosphorylation sites mediated by Mec1, Rad53, Cdc28 -Clb2, and ubiquitylation on position K1139 [68].

Rad9 protein binds to methylated histone H3 on nucleosomes in undamaged cells via its tandem tudor domain [69], and Rad9's tandem BRCT domain enhances its concentration at DSBs by interacting with phosphorylated Histone H2A (S119). Rad9 is further recruited to DNA damage sites by the Dpb11 (TOPBP1), which interacts with the 9-1-1 complex that is loaded onto ssDNA-dsDNA junctions [70]. This, in turn, facilitates Rad9 phosphorylation by Mec1, which induces Rad9 multimerization via its BRCT domain and enables Rad9 to recruit Rad53 [71]; thus, Rad9 serves as an adaptor so that Mec1 can phosphorylate Rad53 [72]. The oligomerized Rad9 can also serve as a scaffold for the aggregation of multiple Rad53 molecules, which facilitates Rad53 trans autophosphorylation[73] leading to the subsequent release of Rad53. Rad9 thus controls one branch of the checkpoint pathway, the other of which is controlled by Mrc1, whose role in checkpoint activation is restricted to DNA replication forks. Rad9 can also function as an adaptor for Mec1-mediated Chk1 activation [74]. These observations illustrate that Rad9 serves multiple functions in checkpoint activation at distinct stages of the cell cycle.

As a consequence of G_2/M checkpoint inactivation, irradiated *rad9* cells form microcolonies on agar plates. Such microcolonies accumulate inviable cells due to mis-segregation and loss of chromosomal fragments [75]. Similar phenotypes are also evident after exposure to select chemical DNA damaging agents, including methyl methanesulfonate (MMS), cisplatin, and topoisomerase inhibitors; spontaneous chromosome loss is also observed [76]. Thus, in the presence of either spontaneous or environmentally-induced DNA damage, chromosomal fragments or aberrant DNA replication structures could initiate aberrant recombination events that could lead to higher frequencies of chromosomal rearrangements resulting from homologous (HR) or NHEJ.

RAD9 facilitates the completion of DNA replication initiated during S phase. First, it facilitates chromosomal DNA replication when there are large distances between origins, a function which does not require recombination [77]. Second, it backups *MRC1's* function to extend the length of checkpoint signaling during replication stress [78]. Third, it is required for recombination-mediated resolution of aberrant structures that are formed in DNA replication. For example, *rad9* exhibits synthetic lethality with *rad27*[79], a mutant defective in the processing of 5' flaps generated during lagging strand synthesis [80]. The hyper-recombination of *rad27* and the synthetic lethality with *rad27* and *rad52* [81] suggest that HR is required to resolve such aberrant structures that would otherwise confer lethality.

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RAD9's function in controlling HR between sister chromatids and homologs, and ectopic recombination between repeated sequences is not fully understood. Depending on the assay to measure HR, *rad9* mutants may exhibit enhanced recombination, decrease recombination, or no effect. In addition, particular phenotypes may depend on whether the assay measures spontaneous or DNA damage-associated recombination or whether the assay is performed in haploid or diploid cells. Our effort to elucidate these apparently contradictory phenotypes will include: 1) summarizing different recombination assays that exhibit *rad9* phenotypes, 2) describing interactions with *RAD9* and recombinational repair pathways, and 3) comparing phenotypes of *rad9* mutants with mutants in other checkpoint genes. Finally, we will present possible mechanisms that may elucidate these phenotypes and future experiments.

3. Hr Phenotypes of Rad9 Mutants

The *RAD9* requirement for HR and NHEJ events is shown in Table 1. The arrangement of repeated sequences that generate different chromosomal rearrangements is shown in Figure 2. Factors that may affect rates of HR include the size and orientation of the repeats, the distance between repeats, and whether the repeats are identical or divergent. Additional factors in Rad$^+$ strains also include ploidy and whether both *MAT***a** and *MAT*a are expressed.

Table 1. Recombination phenotypes of *rad9* mutants.

Genetic Assay	DNA damaging agent and assay	*rad9* phenotype[1]	Ploidy specificity	Reference
Intrachromatid recombination				
SSA between homeologous repeats	HO-induced DSBs	Decreased	Haploid	[22]
SSA	HO-induced DSBs	NC	Haploid	[94]
Deletions at the rDNA locus	Spontaneous	Increased in *orc1-4* mutants	Haploid	[92]
Sister chromatid recombination				
uSCR	Spontaneous	NC	Haploid	[82]
uSCR	X rays	Decreased	Haploid	[82]
uSCR	MMS	Decreased	Haploid	[82]
Equal SCR	1-Sce1 induced break	Decreased	Haploid	[83]
Homolog recombination between heteroalleles				
Homolog recombination occurring between two heteroalleles	Spontaneous	Two-fold increase	Diploid	[101]
Allelic recombination	Spontaneous	MC	Diploid	[100,117]
Gross chromosomal rearrangements (GCRs)				
GCR	Spontaneous	Increased	Haploid	[119,123,127]
GCR	Spontaneous	Increased	Disome for VII	[117]
Recombination between repeats on non-homologous chromosomes				
Directed translocations	Spontaneous	Increased	Haploid and Diploid	[82]
Directed translocations	Radiation (X-ray, UV)	Increased	Diploid	[82]
Directed translocations	Topoisomerase Inhibitors	Increased	Diploid	[89]
Directed translocations	MMS	Increased	Diploid	[82,89]
Directed translocations	4-NQO	No change	Diploid	[89]
Directed translocations	HO-induced breaks	No change	Diploid	[82]
Chromosoe III translocations	Spontaneous	Increased in orc1-4 mutants	Diploid	[104]

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Ectopic Gene Conversion	Spontaneous	No Change	Haploid and Diploid Strain	Fasullo (unpublished)
Non-homologous end-joining between cohesive ends				
Chromosome Breaks	HO-induced breaks	Decrease	Haploid	[94,122]
Plasmid Breaks	pRS315 digested with BamH1	Decrease	Haploid	[121]

[1]Rate or frequency in comparison to wild type, see reference for more detail.

3.a. RAD9 Requirement for SCR

Recombination between sister chromatids proceeds by multiple mechanisms, including template-switching and DSB-initiated HR. While *RAD9* is not required for spontaneous sister chromatid exchange, *RAD9* is required for DSB-associated sister chromatid recombination as demonstrated in assays to measure either equal or unequal sister chromatid recombination [82,83]. The assertion that X ray-associated recombination requires a G_2/M checkpoint is supported by observations that rad9's X-ray sensitivity is suppressed when cells are irradiated after pretreating with the microtubule inhibitor nocodazole. Both X-ray and HO-induced DSBs do not stimulate as many uSCR events in *rad9* mutants as they do in wild type [82,83] have shown that *RAD9* is also required for DSB-initiated equal SCR in a pathway that involves stabilization of cohesin; cohesin is absolutely required for repair of DSBs in yeast [84] and participates in the DNA damage response [85]. This observation is further supported by observations that one downstream effector of Mec1 activation, Rad53, which contributes to the cohesin stabilization and maintenance of cell cycle arrest, is required for X-ray associated SCR [86]. While Chk1 is also a downstream effector of Mec1 activation, *chk1* mutants are not X-ray sensitive [87], nor do they exhibit a clear defect in sister chromatid cohesion [88] or X-ray associated uSCR [86]. These results suggest that Rad53 plays a major role in the *RAD9*-mediated checkpoint pathway that facilitates DSB-associated SCR.

rad9 mutants exhibit fewer DNA damage-associated uSCRs after exposure to selective chemical agents [25,82,89], particularly those that indirectly generate DSBs, such as methyl methane sulfonate (MMS) and camptothecin, a topoisomerase inhibitor. However, *rad9* mutants exhibit only a minor decrease in UV-associated uSCR [82] and no decrease in the 4-nitroquinoline 1-oxide (4-NQO)-associated uSCR events [89]; 4NQO is a UV-mimetic agent. Since UV promotes *RAD5*-dependent template switch events [90] , and both UV and 4-NQO-associated uSCR require *RAD5* [91], these observations suggest that *RAD9* is not required for template switching.

3.b. rDNA Repeat Instability and CNV

While *RAD9* promotes DSB-associated SCRs, it suppresses rDNA repeat instability that results from insufficient DNA replication firing due to non-functional origin recognition complex (Orc) proteins, as present at the restrictive temperature in *orc2-1* mutants [92]. *RAD9* confers lethality in *orc2-1* diploid mutants at the restrictive temperatures. However, the viable colonies obtained in *rad9 orc2-1* diploid mutant contain reduced numbers of rDNA repeat units. The authors suggest that because rDNA contains many Orc2 binding sites, limited amounts of Orc2 protein at the restrictive temperature are insufficient to initiate replication from other chromosomal origins, and that rDNA may be particularly vulnerable to recombinogenic lesions [68]. Such recombinogenic lesions could promote uSCR, unequal homolog recombination, and intrachromatidal recombination. Reduced rDNA copy number thus allows the initiation of DNA replication at other chromosomal origins and thus confers viability.

Copy number variation (CNV) has also been measured in checkpoint mutants containing multiple juxtaposed *CUP1* repeats (Table 2). In these haploid strains, nicotinamide-mediated suppression of the histone deacetylase H3K56ac induces *CUP1* transcriptional induction initiating a replication fork impediment and CNV contraction. CNV contraction is *RAD52*-dependent and proceeds through HR. Interestingly, *MRC1*, not *RAD9* suppresses CNV [93]. In addition, *rad27* mutants exhibit enhanced CNV, suggesting aberrant replication intermediates are leading to

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recombinogenic lesions. Considering that the double *mus81 yen1* mutant defective in Holiday junction resolution does not exhibit CNV, an attractive mechanism is that *CUP1* transcriptional induction induces replication fork cleavage in S phase initiating a replication restart mechanism. Thus, *RAD9* can suppress instability at the rDNA locus while facilitating instability at *CUP1* repeats [93].

Table 2. Fold Stimulation of Recombination-directed and Gross Chromosomal Rearrangements Generated in *rad9*, checkpoint, and *rad* mutants.

Genotype	*RAD52*-Dependent Rearrangements	Gross Chromosomal Rearrangements (GCRs)			
	his3-repeat-directed translocations[1]	*CUP1* Copy Number Variation(CNV)[2]	yel069c::*URA3* *CAN1*[3]	yel072w::URA3 *CAN*[4]	CenVII *ade6 ADE3*/ *hxk2:CAN1* cenVII *ADE6* *ade3*[e.]
WT	1	1	1	1	1
rad9	7	0.8	6	1.9	23
mrc1		4.1	2	19	4.2
rad53 sml1	10	1	27	16	42
rad5	3.3	0.9	127	19	27
rad27		2.1	1100	140	
rad52	<0.1	0.08	126	0.6	73
mec1-21	23				
mec1-21 rad51	100				
mec1 sml1		1	194	7.6	97
mec1 sml1 rad51			1271		

[1] Rate of spontaneous recombination in diploid wild type is 3 x 10^{-8}, Fold increase is the (rate in mutant)/(rate in wild type). [2] CNV is measured by Southern blot by comparing the intensity of variation in wild type with that of the mutant. [3] Rate of spontaneous GCRs in haploid wild type is 3.5 x 10^{-10}, Fold increase is the (rate in mutant)/(rate in wild type). [4] Rate of GCRs in haploid wild type is 2 x 10^{-8}, Fold increase is the (rate in mutant)/(rate in wild type). [5] Rate of GCRs is Chr VII disome is 2.3 x 10-5, Fold increase is the (rate in mutant)/(rate in wild type).

3.c. RAD9 Is Not Required for SSA Unless There Are Significant Mismatches Between Annealing Sequences

RAD9 is not required for the completion of SSA when an enzymatic-induced DSB initiates recombination between two non-tandem repeats. These assays included one strain that contained tandem *his3* fragments, where an HO endonuclease cut site (HOcs) was inserted in one *his3* fragment, or an HOcs was inserted between *ura3* repeats [94]. However, *RAD9* is required for intrachromatidal recombination between homeologous repeats that contain 3% divergent DNA [85] in which an HOcs was inserted between the 200bp *ura3* repeat units. The *RAD9*-dependence of SSA is suppressed by nocodazole, suggesting that the critical factor for completing recombinational repair between divergent repeats is maintenance in G_2 [95]. The authors suggest that SSA between heterologous repeats requires mismatch repair proteins, which require an extended G_2. While *RAD9* is not explicitly required for SSA, other checkpoint proteins, such as Mec1, do affect SSA by phosphorylating Slx4 [96] and affecting Rad1/Rad10 cleavage on non-homologous 3′ tails [97]. Thus, while checkpoint proteins regulate SSA, the *RAD9* function has not been explicitly defined.

3.d. RAD9 Affects the Outcome of DSB-induced Homolog Recombination

The overall conclusion of *RAD9*-dependence of homolog recombination is that *RAD9* is not required for spontaneous recombination but does alter the type of DSB-induced recombination event. Several strain constructs used to measure rates of spontaneous homolog recombination include those that 1) simply measure gene conversion events between two heteroalleles and 2) those that measure

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cross-overs between two heteroalleles of two or more gene. In the first type of construct, *RAD9* is not required for spontaneous homolog recombination between heteroalleles in either diploids or in a haploid disomic from chromosomes VII [98–100]. In the second strain construct, Can^R and Thr^+ recombinants were selected in a diploid that was heterozygous with wild type at both *CAN1* and *HOM3*. The authors observed a two-fold increase in rates of spontaneous homolog recombination [101]. Thus, *RAD9* has a minimal effect on recombination between homologs.

However, *RAD9* does alter the types of recombination events that are induced by an I-Sce1 restriction endonuclease when the endonuclease recognition site is placed in one copy of an *ade2* gene in a diploid strain containing two *ade2* heteroalleles flanked by different markers [102]. In this strain construction, gene conversion events, cross-overs, and break-induced replication (BIR) events could be identified by scoring the presence of the *ade2* allele and whether the flanking marker was present. Based on these studies, the authors demonstrated that the *rad9* mutant exhibited a higher percentage of short-track gene conversion and a reduced frequency of break induced replication and cross-over events. By Chip analysis, they also showed that Rad9 limits the Sgs1 and Mph1 helicase binding, suggesting that Rad9 facilitates the recombinogenic repair of DSBs by stable annealing of the recipient and donor strands during recombination [102].

3.e. RAD9 Suppresses Ectopic Recombination That Generates Translocations

RAD9 suppresses translocations generated by HR between *his3* repeated sequences located at centromere-linked loci on non-homologous chromosomes II and IV. Compared to the wild-type diploid, the rate of spontaneous homology-directed translocations in homozygous *rad9* diploid mutants increases by seven-fold [82,100]. A modest but significant increase is also observed in *rad9* haploids, compared to the wild type [82]. The hyper-Rec phenotype of the *rad9* mutant is further enhanced if cells are exposed to radiation; an approximately thousand-fold stimulation was observed after *rad9* diploid mutants are exposed to 15.6 krads [82]. The radiation-associated recombination is suppressed by pre-arresting cells with the microtubule inhibitor nocodazole before UV or X ray exposure. These data suggest that cell cycle delay is sufficient to suppress DNA damage-associated translocations.

Many homology-mediated translocations in *rad9* mutants may result from recombinogenic acentric and centromere-containing chromosomal fragments that are inherited in repeated cells cycles (Figure 4). Consistent with this proposal, many radiation-associated translocations are non-reciprocal events, also referred to as half-crossovers (HCs). Although BIR can theoretically generate these HCs, BIR has not been observed to transverse centromeric DNA [103]. Instead, it is likely that multiple rearrangements generate radiation-associated His^+ recombinants, especially those where the His^+ phenotype is unstable. This interpretation is supported by independent observations that DSBs can efficiently remodel the genome [3] and that chromosomal instability in *rad9* mutants can be initiated at telomeric sites [99], which would subsequently trigger recombination at other loci.

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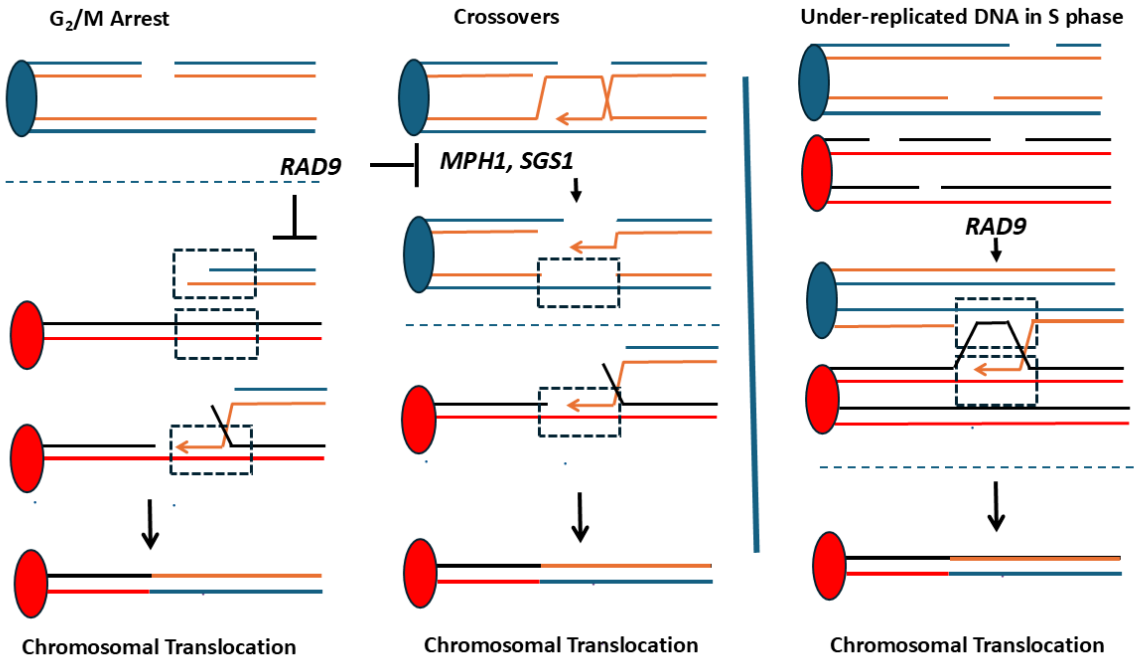

Figure 4. *RAD9* functions in suppressing and promoting homology-directed translocations. The left panel shows mechanisms by which *RAD9* suppresses homology-directed translocations. *RAD9* can promote G_2/M arrest allowing for addition time in the cell cycle to facilitate DSB and DNA repair; otherwise, acentric chromosome fragments are segregated and HR between repeated sequences promotes translocations. In the middle panel, *RAD9* promotes cross-overs by inhibiting helicase function of *MPH1* and *SGS1*, thereby promoting SCR. In the last panel, *RAD9* promotes ectopic recombination in S phase by promoting HR between single-stranded DNA present in repeated DNA sequences.

The higher frequency of DNA damage-associated homology-directed translocations in *rad9* mutants depends on the DNA damaging agent [89]. While DNA damaging agents that directly or indirectly generate DSBs s, such hydrogen peroxide, camptothecin, bleomycin, phleomycin, stimulate more homology-directed translocations in *rad9* mutants, other DNA damaging agents do not. For example, *rad9* diploid cells that are exposed to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) or the UV-mimetic 4-NQO do not exhibit enhanced recombination compared to exposed wild-type cells. These studies suggest that many of these DNA damaging agents trigger recombination between ectopic repeats by mechanisms other than formation of DSBs.

An increase in homology-directed translocations in *rad9* diploids has also been observed in independent studies [104]. In a strain construction to measure loss of heterozygosity (LOH) on chromosome III, where the *URA3* gene is positioned on one chromosome III homolog, *rad9* mutants exhibit a higher frequency of 5-fluoroorotic acid resistant (Ura^-) isolates containing an accompanying chromosomal rearrangement [104]. While the increase in the frequency of spontaneous translocations was a modest threefold, the increase observed in diploid *orc1-4 rad9* mutants was 42-fold. Many of these cross-over events occurred at Ty1 sequences. While the *rad9* mutants did not exhibit an increase in LOH due to gene conversion, LOH events due to cross-over events increased three-fold in the *rad9* diploid and 14-fold in the *orc1-4 rad9* diploid mutant, compared to wild type. These data thus support the notion that *rad9* mutants exhibit more ectopic recombination due to cross-over events.

3.f. RAD9 and RAD50 Group Genes Are Separate Pathways For Suppressing Ectopic Events in Diploid Strains

Models for DSB-initiated checkpoint signaling and HR repair of DSBs suggest that there is cross-talk between the two pathways. The Mre11/Rad50/Xrs2 complex, which facilitates DSB recission, is

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required in both DSBs-mediated checkpoint signaling and in DSB repair. Because Rad9 is required for Mec1-mediated activation of *RAD53*, the model would suggest recombinational repair and *RAD9*-mediated checkpoint pathways participate in the same pathway for suppressing homology-directed translocations [105]. Indeed, in haploid mutants *RAD9* is epistatic to recombinational repair [51,106] in conferring ionizing radiation resistance. While *RAD9* suppresses frequencies of spontaneous homology-directed translocations by seven-fold, diploid mutants defective in *RAD9* either *RAD51*, *RAD55*, and *RAD57* exhibit a synergistic (57-78-fold) increase in the frequencies of spontaneous ectopic recombination (Table 3). In addition, diploid mutants defective in *RAD9* in and either *MRE11* or *XRS2* exhibit synergistic (57-fold) increases in spontaneous homology-directed translocations. These studies suggest that *RAD9* and RAD50 group genes that participate in independent pathways for suppressing spontaneous HR between repeated sequences in diploid strains. The lesions that initiate these events, however, are unknown.

Table 3. Synergistic effects of *rad9* and DNA repair mutants on generating homology-mediated translocations.

Genotype of Single and Double Mutant	Recombination Assay	Ploidy	Fold Increase relative to WT[1]	Fold Increase Compared to *rad9*	Fold Increase Compared to single DNA metabolism mutant	References
rad9	Directed translocations	Diploid	7	1	NA	[82,100]
rad9 rad51	Directed translocations	Diploid	57	8	5.3	[100]
rad9 rad55	Directed translocations	Diploid	77	11	6.2	[100]
rad9 rad57	Directed translocations	Diploid	78	11	5.3	[100]
rad9 rad54	Directed translocations	Diploid	55	8	24	[100]
rad9 mre11	Directed translocations	Diploid	57	8	2	[100]
rad9 mec1	Directed translocations	Diploid	6	1	0.3	[100]
rad9	GCR	Haploid	6	1	NA	[110]
rad9 sgs1	GCR	Haploid	213 (3%)	37	9.7	[110]

[1]Wild type strain is the corresponding Rad$^+$ haploid or diploid form which the single or the double mutant is derived from.

One possible interpretation of the synergistic increase in spontaneous translocation in *rad9 rad51* double mutants is that the ectopic recombination between the repeated sequences is mediated by SSA. Mutations in Rad9 confer more ssDNA while *RAD51* inhibits single-strand annealing SSA [107–109]. Since Rad9 inhibits resection of DSBs and Rad51 inhibits SSA, an increase in ssDNA and lack of inhibition to reanneal these sequences may increase recombination events. Similar interactions would also apply to the interaction of *rad9* and the other *rad* mutants. An alternative explanations is that more DNA lesions accumulate in *rad51* mutants, and these lesions are tolerated in *rad9* mutants. At present, we cannot distinguish the two and it is possible that both mechanisms are important in promoting chromosomal rearrangements that occur by single-strand annealing.

3.g. RAD9 and SGS1 Suppress HR Between Divergent Genes and Ty1 Elements

Schmidt and Kolodner [110] observed that *SGS1* suppresses the formation of gross genome rearrangements (GCRs) that occur by recombination between the *CAN1* positioned on chr V and the

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LYP1 or *ALP1* genes, positioned on chr XIV. *CAN1*, *LYP1*, and *ALP1* encode basic amino acid transporters, which share more than 50% sequence identity. The haploid strains contain *URA3* and *CAN1* genes located on the non-essential chromosomal V arm; double selection against both *URA3* and *CAN1* using 5-fluororotic acid (5-FOA) and canavanine, respectively, generates drug resistant isolates containing chromosomal rearrangements. While most FOA^R and Can^R isolates result from NHEJ events, in *sgs1* mutants, the rate of GCRs is increased 22-fold above wild type, while the *sgs1 rad9* mutant exhibited a 213-fold increase above wild type. While only ~3% of these drug-resistant isolates were translocations due to HR between these sequences, no homology-directed translocations were observed in either the *sgs1* or the *rad9* mutants; translocations found in the single mutants are due to NHEJ or micro-homology end joining [110]. These experiments thus indicate that *SGS1* and *RAD9* constitute independent pathways in suppressing GCRs in haploid strains through multiple mechanisms, including homeologous recombination between sequences.

RAD9 and *SGS1* were also observed to suppress DSB-initiated ectopic rearrangements that directly result from BIR. Vasan *et al.* [111] characterized recombinants that resulted from HCs initiated by galactose-induced DSBs at a HOcs positioned on chromosome III in a chromosome III disomic strain. Deletion of *RAD9* and *SGS1* conferred a higher frequency of recombinants that resulted from compromised BIR leading to cascades of genome instability [111]. One interpretation is that *rad9* mutants exhibit greater mis-segregation of chromosomal fragments while Sgs1 serves as an anti-recombinogenic factor that reverses recombination intermediates generated by BIR. Additionally, Rad9 protects replication intermediates from excessive degradation [112]. Thus, aborted BIR (Figure 1) may lead to aberrant recombination events at ectopic loci. Recombination sites involving HC during compromised HC include Ty1 elements. These data thus indicate that *rad9* mutants exhibit both higher frequencies of spontaneous and DSB-associated recombination between Ty1 sequences.

3.h. RAD9 Is Required for Enhanced Genetic Instability Due to HR Exhibited in mec1 Hypomorphs and Promotes Genetic Instability Resulting from DNA Replication Defects

Various mutants defective in the stabilization of DNA replication intermediates accumulate recombinogenic lesions in S phase, due to DNA replication fork collapse, failure to adequately process Okazaki fragments or resulting from lower levels of dNTPs. This is particularly true of *mec1* hypomorphs, such as *mec1-21* and *mec1-srf* mutants [113], which exhibit decreased viability when *RAD52* is inactivated. The hyper-recombination of *mec1-21* is suppressed by mutations in *SML1*, an inhibitor of ribonucleotide reductase, suggesting that elevated dNTP levels reduce the accumulation of recombinogenic substrates [114]. Unlike *rad9* mutants, which do not exhibit higher rates of spontaneous uSCR and heteroallelic recombination, *mec1-21* mutants exhibit hyper-recombination in spontaneous uSCR and heteroallelic recombination. *RAD9* is required for the hyper-recombination phenotypes that are exhibited by *mec1-21* mutants and *mec1-21 rad9* double mutants exhibit similar rates of homology-directed translocations as *rad9* diploids. An attractive model is that *RAD9* is required to delay the cell cycle so that recombinogenic DNA damage produced by replication fork collapse or replication errors can be repaired. However, additional observations indicate that *RAD9* is required to protect stalled or collapsed replication forks from excessive degradation [112], and that *rad9* mutants exhibit hyper-resection leading to Mec1-mediated Sgs1 phosphorylation [115]. Thus, a combination of factors may function to ensure that recombinogenic lesions generated during DNA replication can be adequately repaired so that replication fork progression may be complete.

Phenotypes of *mec1-21 rad9* mutants are partially mimicked by *mec1-21 chk1* and *mec1-21 pds1* mutants, while higher frequencies of homology-directed recombination observed in *rad9* diploids are also exhibited by *rad53* diploids [87]. For example, mutations in either *PDS1* or *CHK1* reduce the hyper-recombination phenotype of *mec1-21*, and also increase radiation sensitivity in *mec1-21*, as observed in *mec1-21 rad9* mutants. One interpretation of these results is that mutations in either *CHK1* or *PDS1* confer toleration under-replicated DNA, which would otherwise be a substrate for HR

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proteins. These observations suggest that *RAD9* functions in promoting genome instability observed in S phase checkpoints function through downstream effectors.

Besides promoting recombination in S phase checkpoint mutants, *RAD9* promotes triplet GAA repeat expansion in *cdc13-1* mutants [116]. These mutants exhibit telomeric single strands and such single strands may sequester Mrc1 and additional proteins required for replication fork signaling. Although slowed replication progression is not associated with GAA repeat expansion in the *cdc13* strain, the *RAD9*-mediated checkpoint pathway genes, *RAD9*, *RAD53*, *MEC1*, and *EXO1* are required. The authors postulate that *RAD9* is required in post-replicative repair and that polymerase replication over such replicative gaps may promote the repeat expansion [116].

4. Rad9 Role in Suppressing Gcrs

RAD9 functions in suppressing gross GCRs that result from fusion of indirect repeated sequences, which does not require HR functions. This was demonstrated in a chromosome VII disomic strain where one copy of chromosome VII contains a telomeric *CAN1* gene and internal short, inverted repeats. Spontaneous Can^R mutants containing rearrangements generated by fusion of these inverted repeats can then be selected and screened based on sectored colony phenotype; sectors typically contain unstable dicentric and acentric chromosomes [117,118]. *rad9* mutants exhibit 17-fold higher frequencies of Can^R isolates containing such rearrangements. The authors suggest that inverted repeat fusions result from aberrant template switching events during S phase.

However, *RAD9* and G_2/M checkpoint genes have a minor role in suppressing spontaneous Chr V rearrangements, compared to S phase checkpoint mutants [119]. The GCR assay used a haploid strain containing *URA3* and *CAN1* genes located on the non-essential chromosomal V arm; double selection against both *URA3* and *CAN1* using 5-fluororotic acid (5-FOA) and canavanine, respectively, generates drug resistant isolates containing chromosomal rearrangements. *rad9* mutants have slightly elevated rates (threefold) of GCRs, while the double *rad9 exol* mutant exhibits a slightly more elevated rate of GCR, compared to the single mutants [120]. Even when *URA3* gene is placed in a position adjacent to Ty1 element, the overall rate of GCRs in *rad9* mutant is only two-fold greater than that observed in wild type (Table 2). Since non-homologous end joining (NHEJ) is a major mechanism for generating GCRs, one possible explanation for the modest increase in GCRs in *rad9* mutants is the *rad9* deficiency in NHEJ [121,122]). Another contributing factor is that *rad9* mutants exhibit chromosome loss [76], which may be lethal in haploid strains.

While *RAD9* function in suppressing spontaneous double drug-resistant isolates is minor, its function in suppressing frequencies of DNA damage-associated GCRs is significant. Cells exposed to 0.07% MMS, exhibit -168-fold more GCRs compared to the spontaneous frequency in *rad9* [123], while wild-type exhibits a 68-fold more GCRs, compared to the spontaneous frequency in wild type. While this fold increase may seem minor, the overall frequencies of MMS-associated GCRs is approximately 1200-fold greater than the frequency of spontaneous GCRs in wild type [123], compared to the 98-fold increase in the frequency of MMS-associated GCRs observed in wild type compared to the spontaneous frequency observed in wild type. MMS is known to significantly impede DNA replication [124], supporting observations that S phase DNA errors are the major initiating cause for GCRs.

Comparing the genetic control and DNA damage-inducibility of GCRs and HR-directed translocations reveals important similarities and differences. The major similarity is that defects in S phase checkpoint defects confer the highest increases in frequencies of either GCRs or HR-mediated rearrangements, likely due to recombinogenic structures generated during replication. Additionally, the higher rates of spontaneous GCRs and HR-directed translocations observed in the *mec1* mutant can be synergistically increased by knocking-out *RAD51* [125], suggesting that the checkpoint and recombinational repair pathway are independent in suppressing both NHEJ-mediated and HR-mediated rearrangements. *RAD9* suppresses both DNA damage-associated GCRs or HR-directed translocations, particularly when cells are exposed to the X-ray mimetic chemical, MMS. Both

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frequencies of GCRs and HR-directed translocations are stimulated by diverse DNA damaging agents, ranging from simple alkylating agents to both X rays and UV.

The difference between the genetic control and DNA damage-inducibility of GCR and HR-directed translocations are also notable. DNA damage-associated but not spontaneous GCRs require yKu70 [123,126,127], a gene required for NHEJ; whereas, the highest frequencies of radiation-associated HR-directed translocations are observed in diploid and not haploid strains [128], in which NHEJ is repressed. Indeed, in *yku70* haploid mutants, DNA damage-associated GCRs are abolished [123] while five to six-fold increase in X-ray associated homology-directed translocations are observed in haploid yku70 mutants [129]. While the increase in DNA damage-associated GCRs is significant, the total number of radiation-associated HR translocations is significantly higher. These observations underscore observations that DSBs are important lesions in remodeling the yeast genome even when NHEJ is abolished.

5. Similarities of Rad9 Orthologs in Promoting Genetic Stability

The budding yeast RAD9 ortholog in *Schizosacharomyces pombe* (fission yeast) is *crb2*$^+$ and the human ortholog is 53BP1, which share structural and functional similarities. Similar to budding yeast Rad9, 53BP1 and Crb2 contain Tudor(2) and BRCT(2) domains, which enable these proteins to bind to chromatin and DSBs and provide a scaffold for additional checkpoint proteins [130].They are also recognized and phosphorylated by cyclin-dependent kinases and ATM, and promote HR-mediated repair of DSBs and cross-overs while enabling checkpoint-mediated arrest. They differ, however, in how they regulate HR and promote NHEJ.

In S. pombe, *crb2* mutants are defective in DSB repair and exhibit enhanced loss of linear mini-chromosomes and LOH, likely due to BIR [131]. This was shown in haploid strains that contain a linear mini-chromosome in which a HO-endonuclease cut site is flanked by a non-tandem duplication, and SSA was measured upon induction of the DSB. In an independent study, the X-ray sensitivity of *crb2* mutant deficient in Cdk-mediated phosphorylation was shown to be suppressed by mutations in topoisomerase III, suggesting that lack of crb2 leads to the accumulation of recombination intermediates [132]. These studies have suggested that *S. pombe crb2*$^+$ may function at multiple stages in HR. However, since diploidy is unstable in S. pombe [133], it is difficult to ascertain to the function of *crb2*$^+$ in suppressing the formation of rearrangements that would confer lethality in haploids.

While 53BP1 is a well-known marker for DSBs in mammalian cells, 53BP1 has opposing roles in promoting HR; while it limits resection and thus promotes NHEJ, it promotes DSB repair by HR at heterochromatin in G_2 cells [134]. The role of limiting end resection, however, serves important in VD(J) recombination and at unprotected telomeres. Thus, both Crb2 and 53BP1 function to promote HR in particular contexts. These studies indicate evolutionary conservations of some functions of budding yeast *RAD9*.

6. Summary

RAD9 plays opposing roles in both promoting genetic instability and suppressing genetic instability. By arresting the cell cycle at the G_2/M stage, *RAD9* provides time for recombinogenic DNA damage to be adequately repaired by SCR and by SSA between sequences that have significant mismatches. On the other hand, by triggering a checkpoint response due to replication errors, *RAD9* triggers downstream effectors that may promote genome instability resulting in both ectopic recombination and repeat amplifications. While *RAD9's* role in controlling DNA damage-associated checkpoint activation has been well-studied, how *RAD9* suppresses recombination due to spontaneous DNA damage [136] is unknown.

At the molecular level, there are two alternative mechanisms by which *RAD9* influences HR: 1) *RAD9* controls cell cycle arrest at particular points in the cell cycle by facilitating Rad53 and Chk1 activation, and 2) *RAD9* promotes cross-overs, both in its role in controlling DNA end resection and

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in its role in inhibiting both Sgs1 and Mph1. How these *RAD9* activities function in the context of ploidy, position of repeat units, sequence divergence, and sequence orientation has yet been fully investigated. Understanding these functions will translate into a better understanding of the role of human 53BP1 in HR and in DSB repair.

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Abbreviations

CNV, Copy number variation
DSB, double-strand breaks
5-FOA, 5-fluororotic acid
GCR, gross chromosomal rearrangements
HC, half crossover
HOcs, HO endonuclease cut site
HR, homologous recombination
LOH, loss of heterozygosity
MMEJ, microhomology-mediated end joining
MMS, methyl methanesulfonate
NHEJ, non-homologous end-joining
4-NQO, 4-nitroquinoline 1-oxide
Orc, Origin recognition complex
SSB, single-strand breaks
SCR, sister chromatid recombination
uSCR, unequal sister chromatid recombination

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