



Genetic instability is prevented by Mrc1-dependent spatio-temporal separation of replicative and repair activities of homologous recombination

Homologous recombination tolerates replicative stress by Mrc1-regulated replication and repair activities operating at S and G2 in distinct subnuclear compartments

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Homologous recombination (HR) is required to protect and restart stressed replication forks. Paradoxically, the Mrc1 branch of the S phase checkpoints, which is activated by replicative stress, prevents HR repair at breaks and arrested forks. Indeed, the mechanisms underlying HR can threaten genome integrity if not properly regulated. Thus, understanding how cells avoid genetic instability associated with replicative stress, a hallmark of cancer, is still a challenge. Here I discuss recent results that support a model by which HR responds to replication stress through replicative and repair activities that operate at different stages of the cell cycle (S and G2, respectively) and in distinct subnuclear structures. Remarkably, the replication checkpoint appears to control this scenario by inhibiting the assembly of HR repair centers at stressed forks during S phase, thereby avoiding genetic instability.

Keywords:

■ DNA checkpoints; homologous recombination; Rad51; repair centers; replication

Introduction

Faithful replication of the complete genome is essential for preventing any loss of genetic information. However, this is not an easy task; in fact, the genetic instability that accompanies tumor progression during early stages is associated with replicative stress [1–3]. A high risk of mutations and genome rearrangements during DNA replication is linked to the replication fork, a highly dynamic nucleosome-free structure with DNA ends and stretches of single-stranded DNA (ssDNA) susceptible to being the substrate of nucleases and DNA processing enzymes. This fragile structure has to deal with a number of obstacles that hamper its advance, such as DNA adducts generated by endogenous and exogenous agents, abasic sites generated spontaneously or by processing damaged bases, incorporation of rNTPs instead of dNTPs, DNA-binding proteins, specific DNA structures (such as G-quadruplexes, hairpins, and DNA/RNA hybrids), compacted chromatin structures, and even other DNA processes like transcription [4–7]. Additionally, unbalanced supplies of dNTPs or histones strongly threaten fork stability [8–10]. Not surprisingly, cells are endowed with different mechanisms to protect, repair, and restart stressed

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Abbreviations:

BIR, break-induced replication; **CDK**, cyclin-dependent kinase; **DSB**, double-strand break; **FoSTeS**, fork stalling and template switching; **HJ**, Holliday junction; **HR**, homologous recombination; **ssDNA**, single-stranded DNA; **MMS**, methylmethane sulfonate; **NHEJ**, non-homologous end joining.

replication forks. Of these, homologous recombination (HR) plays an essential role, and mutations in genes that encode recombination proteins are associated with defective proliferation, genomic instability, and tumorigenesis [11]. Unexpectedly, though, numerous data have shown that the DNA replication checkpoint inhibits HR repair at double-strand breaks (DSBs) and stressed forks [12, 13]. HR inhibition during S phase is in apparent contradiction with the requirement of recombination proteins to protect and repair stressed forks. In light of recent results, I propose that HR responds to replication stress through replicative and repair activities that are temporarily separated via Mrc1-dependent inhibition of the repair HR centers during S phase. In this way, the DNA replication checkpoint would prevent unscheduled and mutagenic repair events caused by the assembly of repair centers at stressed replication forks. In addition to resolving the aforementioned paradox, this model provides a new scenario to understand why HR is essential in maintaining genome integrity under replicative stress.

Homologous recombination proteins promote the repair of stalled and broken replication forks

The best way to fix DNA is to use an undamaged template, which is the rationale behind HR. The mechanisms of HR use intact homologous DNA molecules to repair DNA breaks, thus preserving genetic information. This is achieved by invading the donor molecule with the 3'-end of the broken molecule. HR has been most extensively studied during DSB repair [14], in which the 5'-end of the broken molecule is resected, generating a 3'-ended ssDNA fragment that is then coated by the ssDNA binding complex RPA (Box 1). The mediator proteins Rad52 and BRCA2 (see Table 1 for yeast and vertebrate orthologs involved in HR and S-phase checkpoints) compete with RPA to load Rad51 (RecA in bacteria), forming a nucleofilament that invades a homol-

ogous sequence. This invasion step generates a D-loop structure that is further stabilized by DNA synthesis and Rad51-mediated strand exchange, events that can lead to gene conversion if the template is not identical. After this critical step, a recombination event can occur by different mechanisms that may or may not involve reciprocal exchange of DNA between the two molecules (crossover) (Box 1).

The breakage of stressed replication forks is an important source of DSBs. HR might wait for the oncoming fork to generate a two-ended DSB or, alternatively, the one-ended DSB generated at fork breakage can be used to restart replication (Fig. 1, steps 1–3). HR-dependent restart of broken forks has been well established in bacteria [15] and seems to also operate in eukaryotes [9, 16–18], for which it is clear that cells are able to initiate replication from an induced DSB (break-induced replication, BIR) [19] (Box 1). In BIR, the 3'-ended molecule can invade a homologous DNA sequence, located either in an allelic or an ectopic position, and prime DNA synthesis for hundreds of kilobases. This occurs through a mechanism that requires all essential DNA replication factors – DNA polymerases and helicases – except the origin recognition complex (ORC) and the kinase Cdc6, which are required to assemble a pre-replication complex [20]. It is therefore possible that a similar mechanism might operate for the restart of broken forks using the sister chromatid as a template.

DSBs are not the only source of recombinogenic DNA damage. In fact, most spontaneous HR events are initiated by non-DSBs DNA lesions [21] that are likely associated with replicative problems [22]. These problems can be due to a reduction in the available pools of dNTPs or defects in DNA synthesis processivity, which can be induced with drugs like hydroxyurea or aphidicolin, respectively. Alternatively, replication forks can stall or collapse – the replisome is disassembled – without breakage when they encounter DNA blocks (e.g. UV light-induced DNA photoproducts, alkylated bases, abasic sites, or DNA-binding proteins), which uncouple the processes of DNA unwinding and synthesis. These situations lead to an accumulation of ssDNA at the fork,

which trigger different mechanisms to overcome the problem. These mechanisms, which have been studied in detail in response to UV light and alkylating agents like methyl-methane sulfonate (MMS), facilitate the replication fork restart downstream of the lesion and to fill in the ssDNA gaps, prioritizing DNA replication over the repair of the initial lesion [23]. For this reason, they are referred to as DNA damage tolerance mechanisms. HR proteins play essential roles both in response to replication inhibitors and in the DNA damage tolerance response by processes that remain unclear. In mammalian cells, Rad51 and BRCA2 are essential for replication forks to advance in both unperturbed and stressed conditions [24, 25]. In yeast, Rad51 and Rad52 are dispensable for normal replication but are required for replication fork progression through DNA adducts [12, 26, 27], and cells accumulate ssDNA fragments at the forks in their absence [28, 29]. Thus far, different mechanisms have been proposed to explain how HR can promote the stabilization and advance of stressed forks. The invasion and strand-exchange activities of Rad51 might bypass a lesion by invading the intact sister chromatid (Fig. 1, step 4), as suggested by the accumulation of Rad51-dependent HJ-like structures in yeast mutants that are defective in HJ dissolution [30]. Likewise, Rad51 might promote fork reversal by annealing the nascent strands (Fig. 1, step 5), as shown for RecA *in vivo* and for human Rad51 *in vitro* [31, 32]. Additionally, fork reversal can be promoted by helicases (e.g. RecQ and SMARCAL1) [33, 34]. In any case, fork reversal would allow the replication-blocking lesion to be repaired, the lesion to be bypassed by DNA synthesis and regression, or the fork to be restarted downstream of the lesion by strand invasion and HJ formation (Fig. 1, steps 6, 7–8, and 9–10, respectively). As an alternative to these fork stalling and template switching (FoSTeS) models, Rad51 might also bypass the obstacle by recruiting translesion-synthesis polymerases able to incorporate dNTPs opposite DNA adducts, as supported by the physical and functional interactions between human Rad51 and Pol η under conditions of replicative stress [35, 36] (Fig. 1,

Box 1

Mechanisms of DSB-induced homologous recombination

DSB repair by HR is initiated by resecting the 5'-ends of the break to generate the 3'-ended ssDNA molecules, which are common intermediates in all recombination processes. DNA resection is initiated by the Mre11-Rad50-Xrs2/Nbs1 (MRX/N) complex and Sae2/CtIP that, together with BRCA1, counteract the inhibitory activities of the NHEJ Ku complex and the checkpoint protein 53BP1. Then, the nuclease Exo1 and the helicase–nuclease Sgs1–Dna2 extend DNA resection.

The mediator proteins Rad52 and BRCA2 compete with the ssDNA binding complex RPA to load Rad51 and form a nucleofilament involved in the search and invasion of a homologous sequence. This invasion leads to the formation of a D-loop structure that is further enlarged by DNA synthesis and Rad51-mediated strand exchange. Once this recombination intermediate is formed, HR can proceed through different mechanisms.

- (1) Synthesis-dependent strand annealing (SDSA). The strand exchange intermediate is reverted, and the newly synthesized molecule reannealed with the other 3'-ended ssDNA molecule. SDSA can also occur after the second 3'-ended ssDNA molecule has been captured by the D-loop; in any case, the product is a non-crossover.
- (2) Double-strand break repair (DSBR). The D-loop reaches the resected 5'-ends to generate two Holliday junctions (HJ). These can be dissolved by a helicase–topoisomerase complex (leading to non-crossover) or resolved by specific nucleases (leading to either non-crossover or crossover, depending on whether the HJs are cleaved in the same or opposite orientation).
- (3) Break-induced replication (BIR). The second 3'-ended ssDNA is not captured, while the invading 3'-end primes DNA replication through conservative DNA synthesis.

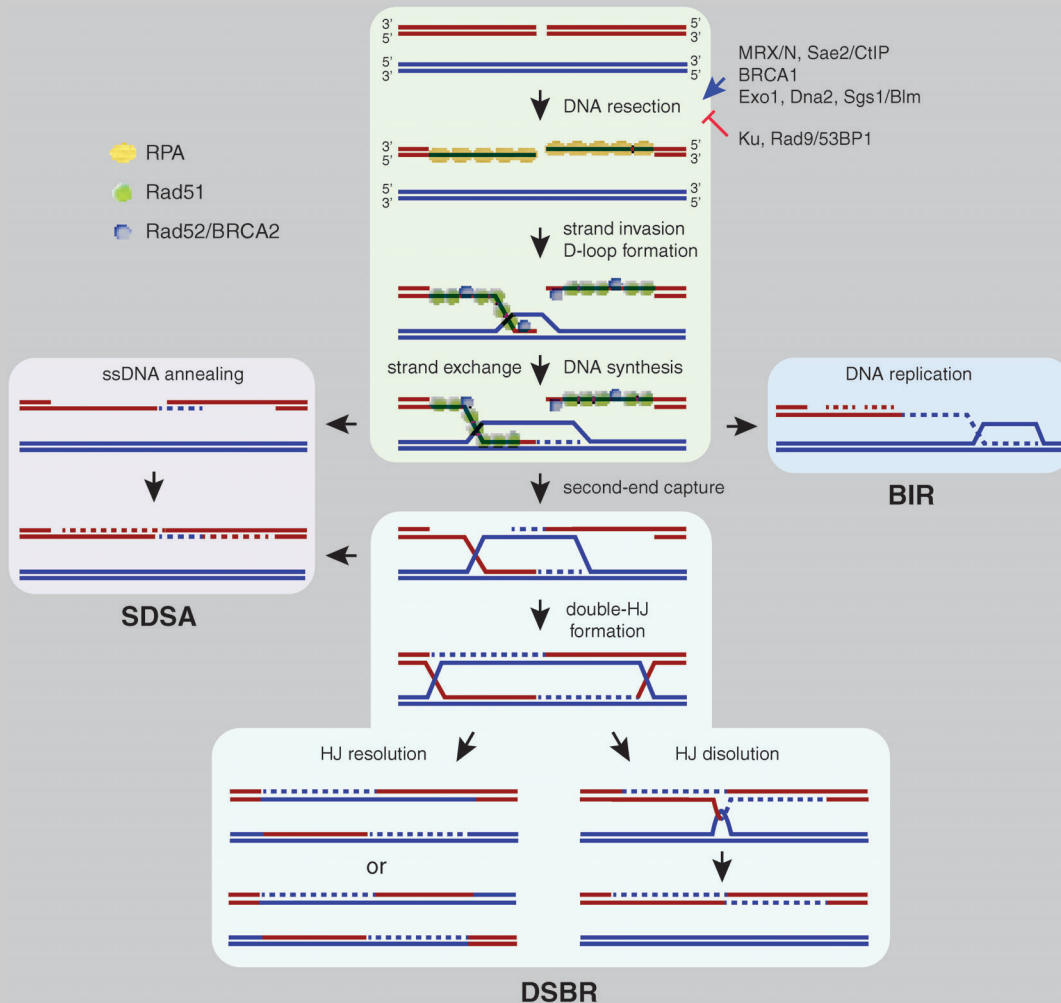


Table 1. Proteins involved in homologous recombination and S phase checkpoints^a

<i>S. cerevisiae</i>	<i>S. pombe</i>	Vertebrates	Function
Rad51	Rhp51	Rad51	DNA strand exchange
Rad52	Rad22	Rad52	Rad51-ssDNA assembly
		BRCA2	Rad51-ssDNA assembly
RPA	RPA	RPA	ssDNA binding
Mre11	Rad32	Mre11	DNA resection
Rad50	Rad50	Rad50	DNA resection
Xrs2	Nbs1	Nbs1	DNA resection
Sae2	Ctp1	CtP1	DNA resection
		BRCA1	DNA resection
Exo1	Exo1	Exo1	DNA resection
Dna2	Dna2	Dna2	DNA resection
Sgs1	Rqh1	RECQ1	Fork reversal/regression
		BLM	Fork reversal/regression
		SMARCAL1	Fork reversal/regression
Mus81	Mus81	Mus81	Fork/HJ-like structure cleavage
Mec1	Rad3	ATR	Checkpoint sensor
Tel1	Tel1	ATM	Checkpoint sensor
Rad9	Crb2	53BP1	DNA damage checkpoint mediator
Mrc1	Mrc1	Claspin	DNA replication checkpoint mediator
Chk1	Chk1	Chk1	Checkpoint effector
Rad53	Cds1	Chk2	Checkpoint effector

^aOnly the proteins – orthologs or functional counterparts – and functions mentioned in the text are shown. *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*.

step 11). In addition, human Rad51 is required to restart replication after hydroxyurea-mediated dNTPs depletion by inhibiting the nuclease activity of Mre11 [24], similar to the nuclease-inhibitory activity proposed for RecFOR in bacteria [37]. These results suggest a scenario in which recombination proteins facilitate replication by protecting the fork. Finally, the “chickenfoot” structure generated by fork reversal could also be cleaved by specific endonucleases to generate a broken fork [38] that would be repaired by BIR-like mechanisms (Fig. 1, step 12).

Homologous recombination proteins escort replication forks

Regardless of the mechanism, recombination proteins might be directly targeted to stressed forks, or they might travel with unperturbed forks to act once they encounter the obstacle. Using a molecular approach to directly follow the binding of recombination proteins to the fork, my group has recently shown in *S. cerevisiae* that Rad52 and Rad51 bind to unperturbed forks, and that this interaction is not increased by DNA damage [27]. Since none of these

proteins is required for DNA replication under unperturbed conditions in yeast [26], we proposed that Rad52 and Rad51 escort the fork to promote DNA replication through damaged DNA [27]. Supporting the idea that this escort function is conserved, Rad51 has been shown to be associated with unperturbed replicating chromatin in human cell lines and in *Xenopus* egg extracts [17, 29, 39], and the absence of Rad51 in yeast and *Xenopus* leads to an accumulation of ssDNA at the fork that is independent of exogenous DNA damage [29]. In fact, different proteomic approaches using human cell lines aimed at isolating factors enriched at or in the proximity of replication forks have recently revealed the presence of the recombination proteins Mre11 and Rad50 [40, 41]. Apart from these components of the core recombination machinery, other factors involved in processing the fork that likely collaborate with HR have been found to travel with the fork in unperturbed conditions. This is the case for the helicase SMARCAL1, which colocalizes with replication factories and helps rescue stalled forks by promoting fork reversal and regression [42]. Notably, homologous recombination proteins are not the only repair factors that travel with the fork; other replication fork escorts reported so far

include the mismatch repair proteins Msh2, Msh3, and Msh6 [40, 43], the DSB repair proteins Ku70 and Ku80 [41, 44], and the single-stranded DNA repair protein PARP1 [44].

Homologous recombination as a source of replication-associated genetic instability

Using a homologous DNA sequence as an information donor for lesion repair makes HR a “safer” fork restart mechanism than other processes. In the case of DSBs, an alternative pathway is non-homologous end joining (NHEJ), where the two ends of the broken molecules are ligated [45]. However, NHEJ usually requires processing of the ends and, consequently, causes a loss of genetic information. Additionally, NHEJ cannot deal with one-ended DSBs generated by breakage of the replication fork. Likewise, template switching by HR provides an error-free mechanism of DNA damage tolerance as compared with translesion synthesis (TLS), an error-prone process in which the DNA lesion that hampers fork progression is bypassed by incorporating a nucleotide opposite to the lesion [46].

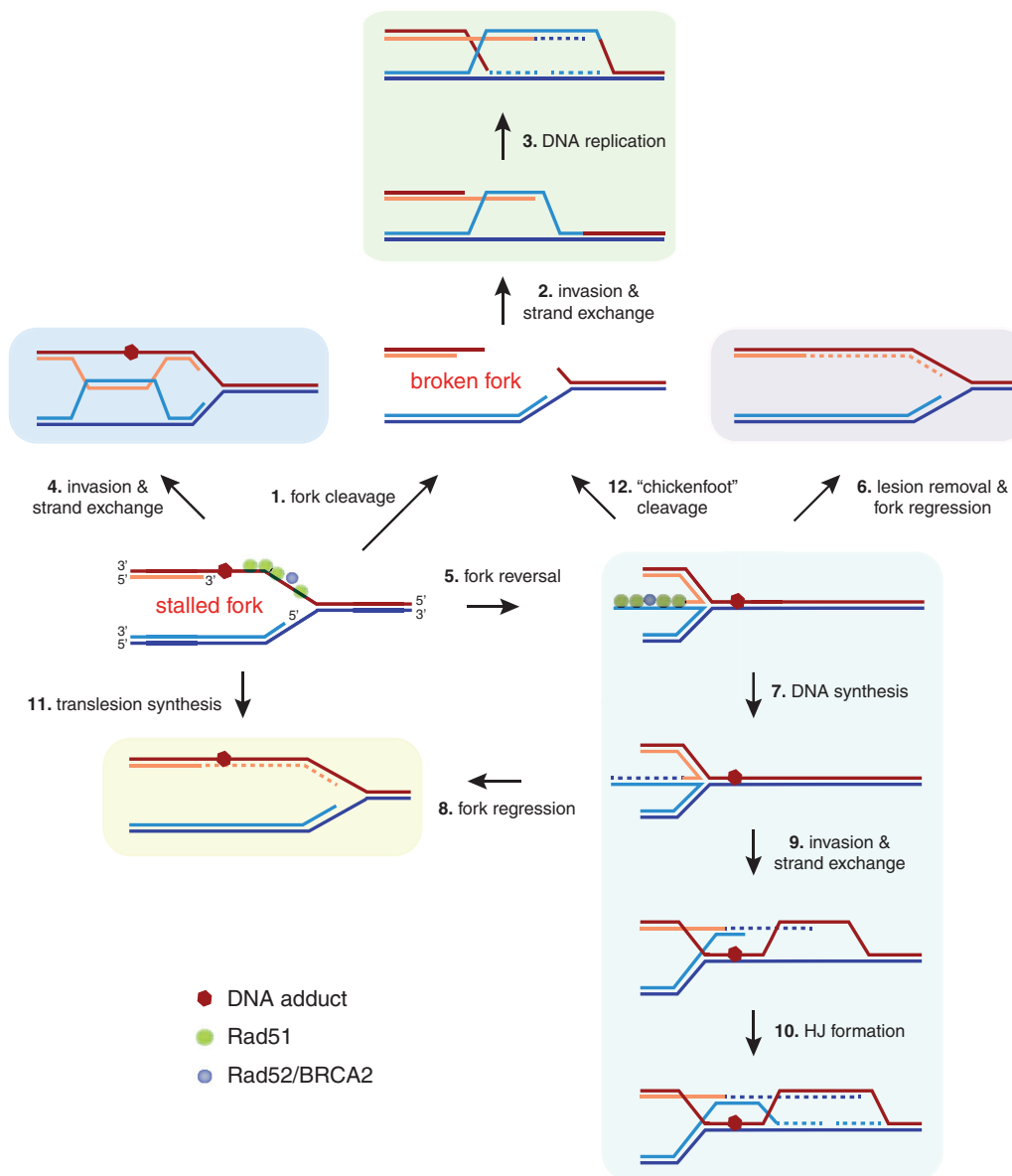


Figure 1. Mechanisms of replication fork restart by homologous recombination. Replication forks can stall or break under conditions that impair their normal progression. At stalled forks, the 3'-ended nascent strand might invade the sister chromatid to generate either an HJ-like structure to bypass the blocking lesion (step 4) or a "chickenfoot" structure by fork reversal (step 5). Fork reversal would allow the replication-blocking lesion to be repaired, the lesion to be bypassed by DNA synthesis and regression, or the fork to be restarted downstream of the lesion by strand invasion and double HJ (steps 6, 7–8, and 9–10, respectively). Apart from these fork stalling and template switching (FoSTeS) mechanisms, Rad51 might bypass the blocking lesion by recruiting translesion-synthesis polymerases able to incorporate dNTPs opposite DNA adducts (step 11). An alternative but not mutually exclusive mechanism might facilitate fork restart by preventing nuclease-mediated degradation of stressed forks (see forks coated with Rad52/BRCA2 and Rad51). At broken forks, the one-ended DSB generated by fork breakage might be resected and invade the sister chromatid, generating a HJ behind the reassembled replisome (steps 1–3). A similar BIR-like mechanism would be required if the "chickenfoot" structure generated upon fork stalling is cleaved by specific endonucleases (step 12). Dashed lines indicate newly synthesized DNA.

However, despite the fact that it is essential for the restart of stressed and broken forks, HR involves a risk for genome integrity. First, DNA synthesis during both two-ended DSB-induced HR and BIR is highly mutagenic as compared with normal DNA replication

(1,000–2,000 times higher) [47, 48]. In the case of BIR, this inaccuracy is associated with an accumulation of ssDNA behind a migrating bubble that drives conservative DNA synthesis [49–51]. Therefore, fork restart by replisome reassembly may represent

a safer mechanism than BIR-like processes.

Second, HR repair involves the formation of replication intermediates and intermolecular junctions that have to be properly resolved in order to prevent genetic instability. In the

absence of activities involved in the formation and resolution of recombination intermediates (e.g. the RecQ helicases or the nuclease Mus81), unrestrained HR causes genome rearrangements [22, 52, 53].

Third, HR is a potential source of genetic instability when it does not go through an equal sister-chromatid recombination event. It can lead to loss of heterozygosity, an event that can uncover deleterious mutations. In addition, HR can generate copy number variations (CNV) – amplifications and deletions – as well as inversions and translocations when the information donor is an ectopic DNA repeat located in the same chromosome, a non-homologous chromosome, or even the sister chromatid (Fig. 2). Even though these chromosomal rearrangements can occur through two-ended DSB repair mechanisms, they are expected to mainly occur by ectopic BIR and FoSTeS processes during replicative stress. In fact, BIR and FoSTeS seem to account for some of the CNVs and complex

genomic rearrangements associated with cancer and genetic disorders [54–57]. Two major features of BIR, which might be formally extended to FoSTeS, can explain their highly deleterious effects on genome integrity. First, BIR can proceed several rounds of strand invasion, DNA synthesis, and dissociation, and this can lead to gross chromosome rearrangements if it occurs within dispersed repeat sequences [58] (Fig. 2). Second, BIR can proceed through a non-homologous recombination mechanism by invading microhomologous DNA sequences (MMBIR, microhomology-mediated BIR) [59], which might explain the presence of microhomologies at the breakpoints of complex genomic rearrangements that have been associated with genetic diseases and cancer [54, 60].

Studies in yeast have shown that although these events are rare compared to equal sister-chromatid recombination events [61, 62], their frequency is high enough to threaten genome integrity [63], especially if stimulated by agents that impair replication fork

advance. In these cases, fork restart can proceed by template switching events using ectopic DNA repeats [64]. In conclusion, HR has to be tightly regulated to avoid unscheduled recombination events associated with the restart of stressed replication forks, as these events can lead to a dramatic loss of genetic information.

An unresolved paradox: Replication checkpoints inhibit recombinational repair, yet recombination is required to restart stressed forks

How do cells control HR to promote fork protection/restart and to prevent unscheduled and deleterious recombination events? A key mechanism in this regulation is the DNA damage checkpoint, which operates throughout the cell cycle to prevent chromosome

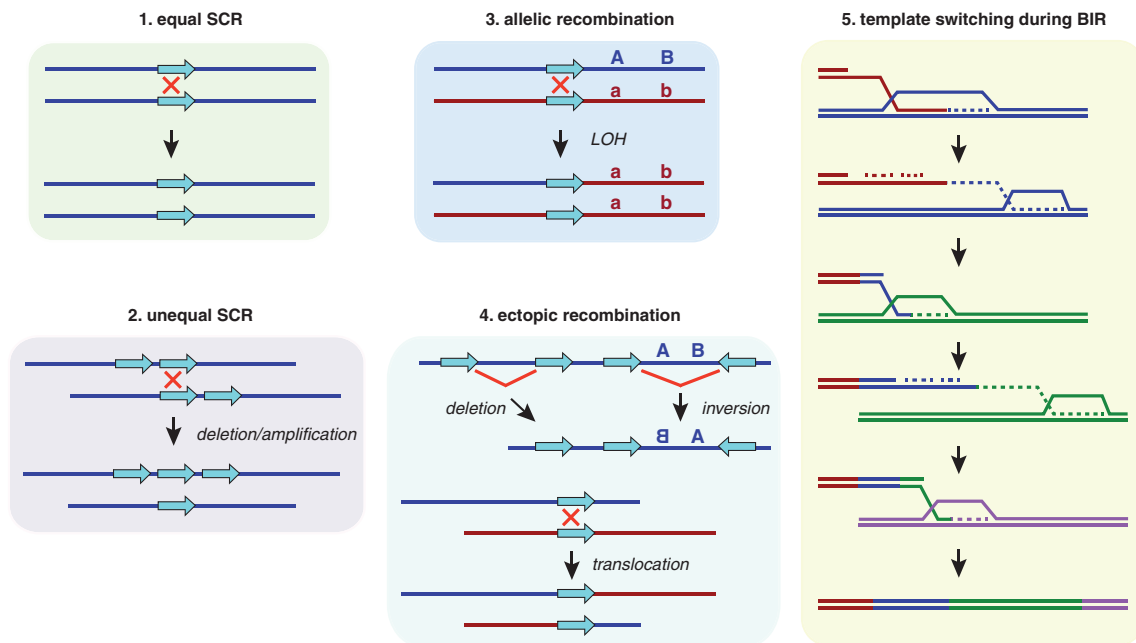


Figure 2. Mechanisms of homologous recombination-mediated genome rearrangements. Unless the sister sequence is used as the template (*equal SCR* (1), equal sister-chromatid recombination), repairing recombinogenic DNA lesions can generate genetic instability. Examples of genome rearrangements associated with crossovers include: *unequal SCR* (2), which leads to deletions and amplifications when it occurs between direct repeats, and acentric and dicentric chromosomes when it occurs between inverted repeats (not shown); *allelic recombination* (3) – between homologous chromosomes – which leads to loss of heterozygosity (LOH); and *ectopic recombination* (4) between repeats located either in different or the same chromosomes in a non-allelic position, which leads to deletions, inversions, and translocations, depending on the position of the repeats. Additional genetic instability can arise by gene conversions as well as one-ended template switching events (such as shown in Fig. 1) through unequal, allelic, or ectopic HR (not shown). Finally, multiple rounds of strand invasion, DNA synthesis, and dissociation during BIR can lead to a particularly catastrophic event if they occur between dispersed DNA sequences (*template switching during BIR* (5)). Dashed lines indicate newly synthesized DNA.

duplication or segregation until damaged DNA is repaired. A number of sensor proteins detect different types of DNA lesions, thereby triggering a phosphorylation-mediated signal cascade that then activates a plethora of responses to coordinate the DNA damage repair/tolerance with cell cycle progression [65]. The DNA damage checkpoint works together with cyclin-dependent kinases (CDKs), master regulators of cell cycle progression, to avoid HR-mediated genetic instability through post-translational modifications of recombination proteins. These modifications prevent unrestrained recombination by regulating the stability and reversibility of critical recombination intermediates (DNA resection, Rad51/ssDNA formation, strand invasion and D-loop formation, and joint molecules resolution) through pro- and antirecombinogenic functions [66].

A key step in the choice of the repair mechanism is DNA end resection, which is tightly regulated during the cell cycle by the CDKs and the DNA damage checkpoint. DNA resection prevents NHEJ and promotes HR [67], but extensive resection inhibits BIR [68]. The Mre11 complex and Sae2/CtIP counteract the DNA resection inhibitory activity of the Ku complex [69], and initiate DNA resection [70–72]. This produces the substrate for further nucleolytic degradation, which occurs in two parallel pathways driven by the nuclease Exo1 and the helicase–nuclease complex formed by Sgs1/Blm and Dna2 [73–75]. CDK-dependent phosphorylation of Sae2/CtIP and Dna2 is required for proper resection [76–78], thus restricting HR to the S and G2 phases of the cell cycle. Additionally, CtIP phosphorylation promotes its interaction with the tumor suppressor protein BRCA1, which is required to recruit CtIP to the break and for further DNA end resection [79]. In contrast, the DNA damage checkpoint protein Rad9/53BP1 negatively regulates DNA resection, the latter by counteracting the resection activities of CtIP and BRCA1 [80]. Since checkpoint activation is triggered by an accumulation of ssDNA [81], this strategy leads to a tight control of the tract of DNA resection, and consequently of the repair mechanism.

In addition to the DNA damage checkpoint, cells are endowed with a

DNA replication checkpoint that operates only during the S phase as it is specifically activated by stressed replication forks [82, 83]. The DNA damage checkpoint and the DNA replication checkpoint are highly interconnected networks that share many signals, components, and responses. Still, they can be genetically distinguished by the mediator protein that connects the two major sensors (Mec1/Rad3/ATR and Tel1/ATM) with the two major effectors (Rad53/Cds1/Chk2 and Chk1) of both pathways: Rad9/Crb2/53BP1 is present in the DNA damage checkpoint, and Mrc1/Claspin in the DNA replication checkpoint [84–88] (Table 1). Mrc1/Claspin travels with the fork and ensures that DNA synthesis and DNA unwinding processes are coupled under unperturbed conditions through a replicative, checkpoint-independent activity; when the forks are stressed, Mrc1/Claspin promotes checkpoint activation by hyper-phosphorylation of the effectors; finally, the Mrc1 replicative activity facilitates fork restart upon block release [85, 89–91]. However, Rad9 can bind to damaged forks in *mrc1* yeast mutants [91], which explains the backup checkpoint activity of Rad9 in the absence of Mrc1 [86]. Besides being involved in general checkpoint responses like inhibition of mitosis and activation of DNA repair mechanisms, the DNA replication checkpoint is specifically aimed at stabilizing stressed replication forks [92, 93]. For instance, checkpoint activation by dNTPs depletion in yeast and mammalian cells phosphorylates the nucleases Exo1 and Mus81 and inhibits replication fork cleavage and genetic instability [94–96]. In mammalian cells, the inhibition of Mus81 and other endonucleases that can process aberrant replication forks is at least in part mediated by ATR-dependent phosphorylation of the fork escort SMARCAL1, which modulates its fork regression activity [97, 98].

Intriguingly, experiments in yeast have shown that a major task of the DNA replication checkpoint is to inhibit HR repair [99]. An important and conserved feature of HR repair occurs through its association with subnuclear foci [100]. HR foci can be detected after treating cells with agents that induce DSBs and therefore activate the DNA damage

checkpoint [101–104]. However, additional activation of the DNA replication checkpoint with drugs that interfere with proper fork dynamics, like MMS or hydroxyurea, suppresses the formation of DSBs-induced HR foci [12, 105]. At the molecular level, hydroxyurea or MMS activation of Mrc1 inhibits DNA resection and thereby the formation of the substrate for Rad52 and Rad51 [12].

The DNA replication checkpoint inhibits not only HR at DSBs but also at forks stressed by dNTPs depletion. Yeast cells treated with hydroxyurea do not form HR foci [102]; intriguingly, studies in *S. pombe* have shown that the formation of HR foci upon hydroxyurea treatment requires cells to complete replication, unless they are checkpoint deficient and thus form persistent HR foci in S phase. At the molecular level, the DNA replication checkpoint prevents the formation of aberrant recombination-dependent structures at the fork [13]. These data suggest that the DNA replication checkpoint promotes a temporal separation of replication and HR by restricting the latter to G2. Accordingly, avian DT40 cells do not form HR repair centers in response to replication-associated DNA lesions until G2 [106]. However, HR inhibition during S phase is in apparent contradiction with the requirement of recombination proteins to protect and help advance stressed forks.

Spatio-temporal regulation of replicative and repair recombination activities

Intriguingly, fork restart after dNTPs depletion in mammalian cells requires Rad51 and BRCA2 but not their repair activity [24]. In fact, human Rad51-mediated restart after a short hydroxyurea treatment is not associated with the formation of HR repair centers, while forks collapsed after a long hydroxyurea treatment do not restart but are repaired at HR repair centers [39]. Both roles of Rad51 in response to replicative stress require BRCA1 and CtIP, which regulate the amount of phosphorylated RPA2 in the case of stalled fork restart, suggesting that BRCA1/CtIP promotes Rad51 recruitment by modulating DNA resection [107]. These results support a role

for the tumor suppressor genes BRCA1 and BRCA2 in HR-mediated restart of stalled replication forks.

This dual role of HR in replication and repair is not limited to the hydroxyurea treatment condition in mammalian cells. Thus, although yeast Rad52 and Rad51 are detected at MMS-induced DNA lesions not only in S phase but also when replication is largely completed, Rad52 and Rad51 foci do not

form until G2. Therefore, lesion repair is not coupled to the fork but in fact prevented during S phase [27]. Since yeast Rad52 and Rad51 are bound to the forks and are required for their advance through alkylated DNA [12, 26, 27], these results indicate that Rad52 and Rad51 tolerate MMS-induced replicative DNA damage by cell cycle-regulated replicative and repair activities. The observation that the Mrc1-branch of the S-phase

checkpoint prevents the assembly of MMS-induced Rad52 foci explains why stressed forks require HR yet at the same time activate a mechanism that inhibits HR: the DNA replication checkpoint inhibits the repair but not the replicative functions of Rad52 and Rad51 [27] (Fig. 3). According to this model, Mrc1 would prevent aberrant Rad51-dependent recombination structures in response to hydroxyurea by inhibiting

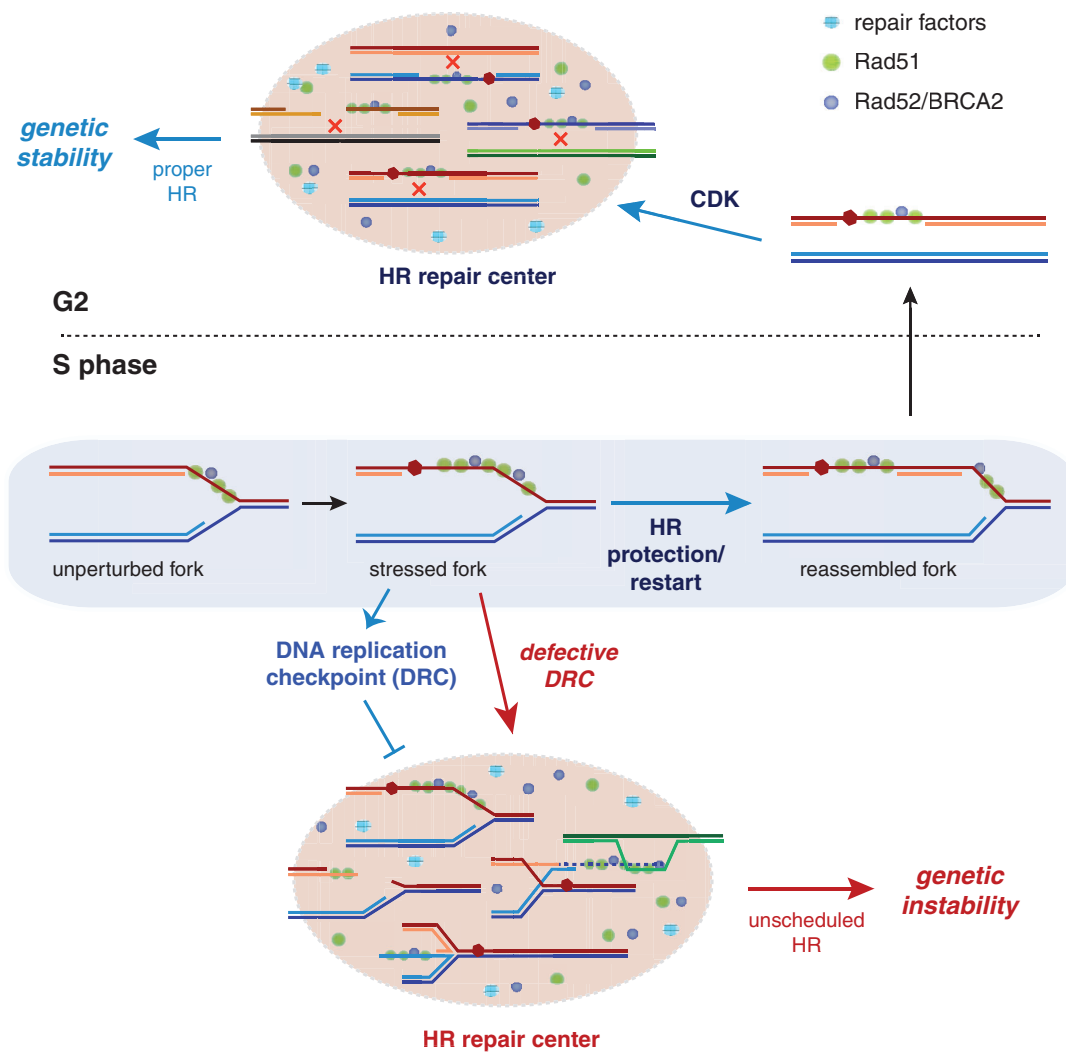


Figure 3. Model for the cell cycle control of recombinational replicative and repair functions by the DNA replication checkpoint. Rad52 and Rad51 bind to unperturbed replication forks. Under conditions that impair fork progression, Rad51 and Rad52 promote the protection/restart of the stressed fork through the reassembly of a canonical replisome (see putative mechanisms in Fig. 1). ssDNA accumulation at stressed forks activates the DNA replication checkpoint (DRC), which prevents the formation of DNA repair centers. Once replication is completed, DRC is switched off, repair centers are assembled through a mechanism that requires the kinase activity of CDK, and recombinogenic lesions are properly repaired. In mutants defective for DRC, the HR repair centers might prematurely assemble at stressed replication forks, thereby interfering with proper DNA replication and promoting genome rearrangements by unscheduled recombination (see putative mechanisms in Fig. 2).

unscheduled repair activities of the recombination proteins traveling with the fork. It is worth noting that the temporal accumulation of MMS-induced HR foci in G2 is not lost in the absence of Mec1 or Rad53 [27]. This observation, together with the fact that Mec1 and Rad53, but not Mrc1, are required for the stability of stressed forks [90], rules out the possibility that the accumulation of HR foci in S phase in *mrc1* mutants is due to replication fork collapse.

While the recruitment of HR proteins to DSBs is independent of DNA replication [12, 105], an important and distinctive feature of the HR response to alkylated DNA is that the binding of Rad52 and Rad51 to the forks during DNA replication is a prerequisite for the further repair of the ssDNA gaps. Thus, if the expression of yeast Rad52 is restricted to the G2 phase, Rad52 cannot bind to the ssDNA gaps, which remain unrepaired and do not form Rad52 foci [27]. Rad52 and Rad51 most likely facilitate bypassing the blocking lesion and then remain bound to the ssDNA gaps left behind the fork until G2 (Fig. 3). The fact that the repair process requires Rad52 and Rad51 binding to the fork indicates that their replicative and repair activities are mechanistically connected although temporally separated.

Replication checkpoint activation by replicative stress inhibits the assembly of the HR centers during S phase [12, 13, 27]. This function, whether or not it is associated with additional mechanisms, ensures that repair does not occur as long as there are stressed forks. Indeed, I think that the logic behind the DNA replication checkpoint-dependent inhibition of recombinational repair is linked to the fact that the repair occurs at HR centers. These centers are highly dynamic structures formed by gigadalton-sized protein assemblies that are able to deal with multiple recombinogenic lesions, likely by concentrating recombination proteins and DNA substrates [100]. This favorable environment for DSB repair might become a threat for genome integrity if the repair centers are assembled at stressed replication forks. Such a situation would interfere with proper DNA replication, which occurs at replication factories [108]. In fact, it might promote fork

restart by a mutagenic BIR process rather than by a safer canonical recombination. Further, the proximity of additional sequences at the repair centers could favor multiple rounds of homology and microhomology-driven BIR and FoSTeS events, which have been proposed to explain single cellular catastrophes (referred to as chromothripsis [109]) that are associated with cancer and genetic diseases [54–57] (Fig. 3).

How the replication checkpoint inhibits the assembly of HR centers is currently unknown. The formation of repair centers in yeast is independent of Rad52 and therefore of any recombination intermediate subsequent to the accumulation of ssDNA [27]. Since ssDNA fragments are already present at the lesions induced by DNA adducts and replication inhibitors, one possibility is that Mrc1 prevents ssDNA extension as a necessary step to form the repair centers. Consistent with this idea, Mrc1 activation by MMS inhibits DNA resection at DSBs [12]. Additionally, the kinase activity of the yeast CDK Cdc28, which is required for DNA resection at DSBs [110, 111], is necessary for the assembly of MMS-induced Rad52 foci [27]. Therefore, the amount of either ssDNA or RPA might regulate the formation of the HR foci. In this context, it is tempting to speculate that the tumor suppressor genes BRCA1 and BRCA2, as well as other factors that control the amount of ssDNA/RPA, might protect against HR-mediated genetic instability by regulating the formation of repair centers.

Therefore, I think that a major task of the replicative checkpoints is to prevent the formation of HR repair centers under replicative stress as they can favor genome rearrangements. An important implication of this function is related to cancer progression. Oncogene-induced alterations in replication dynamics cause an accumulation of replicative stress by unknown mechanisms. This stress leads to checkpoint activation, which act as an early barrier to tumor progression by promoting senescence or preventing genetic instability through DNA repair/tolerance mechanisms. Consistently, mutations that impair this barrier facilitate tumor progression at later stages [1–3]. According to my hypothesis, the assembly of HR centers during oncogene-induced

replicative stress in checkpoint mutants would boost genetic instability.

A mechanistic weakness of this model is the fact that the DNA damage checkpoint, which is activated by DSBs, does not prevent the formation of HR centers despite it operates through the same effectors as the replication checkpoint. Mrc1 might inhibit the assembly of HR centers by different unknown effectors. However, and in contrast to Rad53 in *S. cerevisiae* [27], the *S. pombe* ortholog Cds1 is sufficient to prevent the formation of hydroxyurea-induced HR foci in S phase [13]. Additionally, the mediator activity of Rad9 is able to inhibit DNA resection and HR foci formation in response to replicative stress in a *mrc1^{AQ}* checkpoint-defective mutant [12, 27]. A deeper insight to the many connections between the DNA damage and replication checkpoints in response to different stresses is still missing. In fact, the involvement of Cdc28 in HR foci formation [27] points to a much more complex response that also involves S and M cyclins.

Conclusions

In summary, an important strategy to avoid genetic instability during the rescue of stressed replication forks by HR seems to be to inhibit HR repair centers by the Mrc1 branch of the S phase checkpoints. It is also likely that the DNA replication checkpoint prevents specific recombination intermediates from accumulating at the forks, and this may differ depending on the stress conditions. Once replication is over and the replication checkpoint is turned off, HR centers can be assembled to complete the repair of the accumulated ssDNA gaps and broken ends.

A number of important questions remain to be resolved. The analysis of BRCA2 and Rad51 mutations suggests that the stabilization of Rad51 filaments by BRCA2 is dispensable for DSB repair but required for protecting hydroxyurea-stressed forks from Mre11 degradation [24]. The discovery of a similar dual role for HR in yeast provides a powerful genetic tool to search for additional separation-of-function mutations that help us to define the replicative and repair activities of HR. Additionally, as previously

underlined, future experiments should focus on the relevance of DNA resection in the formation of HR centers. Indeed, we need to advance our understanding of the biology of HR centers as an essential part of the DNA damage response. Although model organisms such as yeast continue to be a helpful tool to approach these problems, parallel studies in higher eukaryotes are necessary for assessing the potential impact of the proposed model in the genetic instability associated with cancer and other genetic disorders. In particular, the regulatory role that Claspin plays in the regulation of DNA repair centers in response to replicative stress should be clarified. Understanding the mechanisms that ensure that the replicative and repair activities of HR occur in distinct subnuclear structures at different cell cycle stages may help us to understand how cells prevent an accumulation of genetic instability during DNA replication.

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