HHS Public Access

Author manuscript

DNA Repair (Amst). Author manuscript; available in PMC 2019 March 01.

Published in final edited form as:

DNA Repair (Amst). 2018 March ; 63: 47–55. doi:10.1016/j.dnarep.2018.01.010.

Collaboration in the actions of recombination-intermediate resolving functions with Brh2 during DNA repair and replication stress in *Ustilago maydis*

Milorad Kojic[1,§], **Mira Milisavljevic**[2], and **William K. Holloman**[1,*]

[1]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY USA

[2]Laboratory for Plant Molecular Biology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Republic of Serbia

Abstract

Cells maintain a small arsenal of resolving functions to process and eliminate complex DNA intermediates that result as a consequence of homologous recombination and distressed replication. Ordinarily the homologous recombination system serves as a high-fidelity mechanism to restore the integrity of a damaged genome, but in the absence of the appropriate resolving function it can turn DNA intermediates resulting from replication stress into pathological forms that are toxic to cells. Here we have investigated how the nucleases Mus81 and Gen1 and the helicase Blm contribute to survival after DNA damage or replication stress in *Ustilago maydis* cells with crippled yet homologous recombination-proficient forms of Brh2, the BRCA2 ortholog and primary Rad51 mediator. We found collaboration among the factors. Notable were three findings. First, the ability of Gen1 to rescue hydroxyurea sensitivity of dysfunctional Blm requires the absence of Mus81. Second, the response of mutants defective in Blm and Gen1 to hydroxyurea challenge is markedly similar suggesting cooperation of these factors in the same pathway. Third, the repair proficiency of Brh2 mutant variants deleted of its N-terminal DNA binding region requires not only Rad52 but also Gen1 and Mus81. We suggest these factors comprise a subpathway for channeling repair when Brh2 is compromised in its interplay with DNA.

Keywords

BRCA2; Blm; Mus81; Gen1; DNA repair; replication stress

[*]To whom correspondence should be addressed: Dr. W. K. Holloman, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065 USA, Tel: 212-746-6510, Fax: 212-746-8587, wkhollo@med.cornell.edu.

[§]Current address: Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11010 Belgrade, Serbia

Author contribution

MK and WKH conceived and designed the project. MK, MM, and WKH performed research and analyzed data. MK and WKH wrote the article.

Conflicts of interest statement

The authors confirm that there is no conflict of interest, financial or otherwise in this work.

1. Introduction

Replication stress, a wellspring of genomic instability, can arise from a number of different sources or conditions [1-3]. When DNA is damaged or when nucleotide pools are depleted replication fork progression can be stalled. Similarly, when replication forks encounter aberrant DNA structures, tightly bound proteins, or R-loops, or collide with transcription complexes, progression can be interrupted and derailed. Countermeasures to reset forks after replication stress are in place, but if not implemented appropriately there can be dire consequences. If the integrity of the fork is compromised, replication could fail to complete, or chromosomes distribution to daughter cells could fail to propagate.

Stalled forks can rearrange structurally to enable replication to proceed [4, 5]. Such rearrangement can involve reversal of the fork to form four-armed DNA intermediates and can employ homologous recombination (HR[1]) between nascent sister chromatids to facilitate procession of DNA synthesis past difficult-to-replicate stretches. Structure specific nucleases contribute to resolution of stalled replication fork and recombination intermediates to help restart replication [6-9]. These nucleases include the Mus81-Mms4/MUS81-EME1 (budding yeast/human) complex, Yen1/GEN1, and the Slx1-Slx4/SLX1-SLX4 complex. All three nucleases contribute to resolution of recombination intermediates during the repair of induced DNA double strand breaks in mitotic cells. In addition they also contribute to processing of failing replication forks and/or resolution of recombination intermediates formed during replication fork recovery. Mus81 overlaps with Sgs1/BLM (budding yeast/human) in processing replication-associated recombination intermediates, which can be toxic in the absence of Mus81 and Sgs1/Blm functions [10, 11]. Sgs1, a RecQ helicase, in complex with Top3-Rmi1/BLM-TOPOIIIa-RMI1-RMI2 (budding yeast/human) acts to process homologous recombination intermediates in DNA double-strand-break repair by catalyzing convergent branch migration and decatenation to resolve double Holliday junctions by dissolution [12-14]. In budding yeast lethality caused by the absence of both Mus81 and Sgs1 is suppressed when homologous recombination is eliminated [10, 15], although suppression by loss of homologous recombination is not implemented in mutants doubly defective in Slx1 and Sgs1 [11]. Yen1 functionally overlaps with Mus81 as evidenced by heightened sensitivity to agents that inhibit replication fork progression and by elevated chromosome loss in the *mus81 yen1* double mutant [16-18]. Suppression of this chromosome missegregation by the absence of homologous recombination suggests that replication-associated recombination intermediates are not resolved in *mus81 yen1* double mutant cells, implying that Mus81 and Yen1 act to process such structures in wild type cells [7, 19].

The basic principles of operation and modes of regulation of these recombination-intermediate resolving enzymes have been elucidated through elegant experiments by a number of laboratories, but the functional contribution of the individual components to resolution of faulty replication-associated structures varies from one organism to another. In budding yeast Mus81 and Yen1 are partially overlapping functionally in processing

[1]The abbreviations used are: HR, homologous recombination; HU, hydroxyurea; MMS, methyl methanesulfonate; UV, ultraviolet light

replication-associated recombination intermediates [19], while in fission yeast, which lacks a Yen1 ortholog, the burden of functional activity falls on Mus81 [20]. In budding yeast mutants lacking Mus81 are sensitive to a number of DNA clastogens, while mutants lacking Yen1 are not [19]. The hierarchy of functional involvement appears reversed in flies where mutants defective in Gen1 have severe hypersensitivities while mutants lacking Mus81 exhibit milder effects [21]. In yeast loss of Slx1 does not lead to problems in coping with replication stress, but in worms loss of SLX1 sensitizes germline cells to UV and camptothecin [22] indicating that SLX1 might be more generally used in replication-associated repair.

We have been interested in the action of recombination-intermediate resolving functions in *Ustilago maydis*, a yeast-like fungus that relies primarily on the BRCA2 ortholog Brh2 as mediator governing homologous recombination [23] rather than Rad52 as is the case in budding yeast. *U. maydis* has the standard complement of known resolving functions including Blm, Mus81, Gen1, and Slx1 [24], but in preliminary studies we noted that their hierarchy of deployment appeared to deviate from the paradigm established in budding yeast. For example, the *blm*Δ *mus81*Δ double mutant combination is not synthetically lethal in *U. maydis* [24] unlike the *sgs1*Δ *mus81*Δ combination in budding yeast [10, 11] or *rqh1*Δ *mus81*Δ combination in fission yeast [20]suggesting that toxic replication-dependent recombination intermediates could be resolved by another factor. Furthermore, while sensitivity of the *blm* mutant to the replication stressor hydroxyurea (HU) was dependent on Brh2 active in supporting homologous recombination, we noted the paradoxical situation of *blm* mutants resistant to HU when expressing certain separation-of–function variants of Brh2 still active in homologous recombination [25]. In view of the unorthodox response of these mutants we performed additional studies aimed at characterizing aspects of the recombination-structure resolving systems in more detail.

2. Materials and Methods

2.1 *Ustilago maydis* strains and genetic methods

Manipulations with *U. maydis*, culture methods, gene cloning and transfer procedures, and survival after DNA damage by UV or HU treatment have been described previously [see [25-27] and references therein]. For survival, in brief cells were diluted to 2×10^7 per ml and then aliquots (10 μl) of serial ten-fold dilutions were spotted on solid medium and irradiated with 254 nm UV light or else were plated on medium containing MMS or HU. Plates were incubated at 28° for 2-3 days until colonies developed. The *blm* mutant used in these studies denoted as *blm-KR* was inactivated by mutation in the Walker A box (K443R) as previously described [24]. Other mutants utilized [25] were deletions in which the open reading frames were entirely eliminated and replaced by cassettes expressing resistance to hygromycin, nourseothricin, or geneticin. Mutants included were in the genes (UMAG gene identifiers noted) for Brh2 (03200), Mus81 (04630), Gen1 (02863), and Slx1 (01857), which were identified in the *U. maydis* annotated genome (UMAG) database [see http://pedant.helmholtz-muenchen.de/] by sequence alignment. All strains were derived from strain UCM350 (*pan1-1 nar1-6 a1 b1*) where *pan, nar, a1* and *b1* indicate auxotrophic requirement for pantothenate, inability to utilize nitrate, and mating type loci, respectively.

Self-replicating plasmids expressing genes encoding Brh2 or derivative driven by the glyceraldehyde-3 phosphate dehydrogenase (*gap*) promoter contained the hygromycin phosphotransferase gene (*hph*) for selection [25]. A self-replicating plasmid with a carboxin resistance variant of the succinic dehydrogenase gene (*sdh2*) for selection and expressing the *rus*A gene under control of the *gap* promoter was described previously [25]. For expressing Gen1, the *gen1* open reading frame was amplified from genomic DNA using LongAmp Hot Start Taq polymerase (New England BioLabs) and placed under control of the *gap* promoter in a self-replicating plasmid with carboxin resistance for selection.

3. Results

3.1 Mus81 and Gen1 contribute to resolving replication-stress-associated structures

Loss of either Blm or Mus81 gene function as previously noted had little effect on resistance of *U. maydis* cells to UV or MMS [24], both of which form lesions impeding replication fork progression. For these studies we used a *blm* loss-of-function allele denoted as *blm-KR* that was made by mutating the critical lysine residue 443 in the ATP-binding loop to arginine (Blm^{K443R}) [24]. However, loss of Blm or Mus81 function sensitized cells to the replication stressor HU, although to different degrees–*blm-KR* was much more sensitive to HU than *mus81*Δ, which was only mildly sensitive (Fig. 1A). By contrast loss of the recombination mediator Brh2 resulted in extreme sensitivity to UV and MMS but caused little effect in resistance to HU as noted previously [24]. Removal of both Blm and Mus81 was synergistic in loss of resistance to UV and MMS, and was at least additive, if not synergistic, in sensitivity to HU (Fig. 1A). This finding reinforces the idea that Blm and Mus81 function independently in alternative pathways for processing aberrant DNA structural intermediates. Loss of Gen1 also sensitized cells to HU, but inactivation of Gen1 and Blm did not result in loss of resistance to UV or MMS, nor cause any further sensitization to HU (Fig. 1A). In fact the *blm-KR gen1*Δ double mutant was even somewhat more resistant to HU than the *blm-KR* single mutant suggesting loss of Gen1 slightly suppresses the Blm deficiency. Loss of Slx1 had little phenotype by itself or in combination with Mus81 or Gen1.

These studies revealed some significant functional differences between *U. maydis* and *S. cerevisiae* resolving activities. Most notable is that while yeast *mus81*Δ mutants are highly sensitive to MMS and UV [28], *U. maydis mus81*Δ mutants are not. Conversely, the *gen1*Δ mutant of *U. maydis* is sensitive to HU whereas the yeast *yen1*Δ mutant is not [17]. Also intriguing was the finding that while yeast mutants lacking Sgs1 (the Blm ortholog) and Mus81 are inviable [10, 11], *U. maydis* cells lacking Blm and Mus81 are viable. Furthermore, whereas in yeast *sgs1*Δ *yen1*Δ cells exhibited a similar HU sensitivity to that observed with *sgs*Δ*1* [19] in *U. maydis* the sensitivity of *blm-KR gen1*Δ was more similar to that of the *gen1*Δ single mutant. Curiously, in *U. maydis* the sensitivity of all *gen1*Δ double mutants to HU, *i.e. blm-KR gen1*Δ*, mus81*Δ *gen1*Δ, and *gen1*Δ *slx1*Δ, was similar to that of the *gen1*Δ single mutant regardless of what was the vulnerability of any of the single mutants, suggesting that the processing of the aberrant DNA structures is channeled through the action of Gen1. Despite these prominent differences there are also similarities between *U. maydis* and *S. cerevisiae* resolving functions. Deletion of *gen1/YEN1* in a *mus81*Δ

background potentiates DNA-damage sensitivity. In addition, that yeast *sgs1*Δ *mus81*Δ is lethal but *sgs1*Δ *yen1*Δ is viable parallels the situation in *U. maydis* in which *blm-KR mus81*Δ is hypersensitive to MMS and UV, while *blm-KR gen1*Δ is only mildly sensitive. These similar features reinforce the idea that Mus81 plays a dominant role in both organisms in resolving pathological structures that arise to block replication fork progression.

To gain further insight into the hierarchy of functional implementation among these factors we wanted to determine the effect of expressing Gen1 from a strong promoter in *mus81*Δ and *blm-KR* mutant backgrounds. Overexpression of Yen1 partially rescues the repair defects of *S. cerevisiae mus81*Δ, but not *sgs1*Δ [19] suggesting that Yen1 can serve as a backup to Mus81 by processing similar DNA structures. *S. pombe* lacks a Yen1 ortholog but expression of human GEN1 partially substitutes for Mus81 and even for Rqh1 in promoting resistance to genotoxins [29].

Ectopic expression of *U. maydis* Gen1 in *mus81*Δ *gen1*Δ restored the phenotype to that comparable to *mus81*Δ as expected indicating that the cloned *genl* gene was functional in complementing the deficiency of *gen1*Δ (Fig. 1B). Remarkable, however, was the restoration of resistance to HU, but not to UV or MMS, when Gen1 was expressed in *blm-KR mus81*Δ (Fig. 1B). This finding suggests that Gen1 is unable to substitute for either Mus81 or Blm in repair of DNA damage but is sufficient to process certain replication stress-associated aberrant DNA structures in the absence of Blm. It was interesting to note that the ability of Gen1 to rescue HU sensitivity of *blm-KR*, although substantial, was dependent on the absence of Mus81 (Fig. 1B). Expressing Gen1 failed to suppress the sensitivity of *blm-KR* single mutant. We observed this response in three independent isolates and so ruled out the trivial explanation that Gen1 was not expressed in this *blm-KR* strain. We also note that the construct used for Gen1 expression utilized a strong constitutive promoter so intracellular levels during the cell cycle could be different from that produced with the endogenous gene.

Given these provisos, one interpretation of this observation is that Mus81 functions with Gen1 in the same pathway of managing replication-associated structures but acts upstream of Gen1. The action of Mus81 ensures the processing of the aberrant structures and creation of DNA intermediates that could be further acted upon by Gen1 only if Blm is present. Alternatively, it is also feasible that if the process is to be properly executed it should start with an involvement of Blm. In the absence of Blm, a more complicated array of unresolved DNA structures would be channeled to Mus81 leading to the presentation of structures that could not be processed further by Gen1. In either case Mus81 would convert the aberrant DNA structures into intermediates unresolvable by Gen1. On the other hand if Mus81 and Blm were unavailable the management of unprocessed replication-associated structures would be effectively achieved by the increased levels of Gen1. Regardless of the details, the results nonetheless point to some ordered interplay between the resolvases, and suggest that Gen1 has an important role in processing replication-associated structures.

3.2 Like Blm, Gen1 is involved in resolution of toxic recombination intermediates generated by replication stress

Of single mutants we tested *blm-KR* and *gen1*Δ had the most severe phenotype when challenged by HU, but the *blm-KR gen1*Δ double mutant was no more sensitive than either

single mutant (Fig. 1A) suggesting an epistatic relationship. These effects could be explained by a combined action of Blm and Gen1 in a common pathway for handling aberrant structures arising from HU-induced replication stress. Toxic DNA intermediate structures generated when replication is stressed by HU poisoning in cells lacking Blm can be prevented from forming by eliminating homologous recombination [25]. This is evident by suppression of the HU sensitivity of the *blm-KR* mutant when the Brh2 gene is deleted (Fig. 2) and [24]. By the same token and in the context of the apparent epistasis, we were interested to learn if Gen1 is, like Blm, involved in processing toxic homologous recombination-induced DNA intermediates formed during replication stress. We observed that the sensitivity of *gen1*Δ to HU could indeed be reversed by eliminating the gene for Brh2 (Fig. 2). These findings suggest that Gen1, like Blm, contributes to processing toxic DNA intermediates formed via homologous recombination when replication is stressed and are consistent with the notion that Blm and Gen1 are involved in the same pathway.

It seems clear that Brh2-dependent toxic intermediates formed in the presence of HU must be processed by both Blm and Gen1, as these two factors are not functionally redundant in this regard. A simple interpretation would be that homologous recombination creates two distinct subsets of potentially toxic intermediates that Blm and Gen1 are required to process separately. Alternatively, it could be that Gen1 operates prior to or upstream of Blm in a fashion that dictates the enhanced need for subsequent involvement of Blm protein. That loss of Gen1 slightly suppresses the Blm deficiency might be taken as support for the latter possibility.

3.3 Homologous recombination proficiency does not always predispose cells to HU sensitivity in the absence of Blm or Gen1

In keeping with the hypothesis of a collaborative role for Blm and Gen1 we examined the response of *brh2*Δ *blm-KR* and *brh2*Δ *gen1* to challenge when different structural variants of Brh2 were present. Brh2 exhibits remarkable plasticity in maintaining functional activity in homologous recombination and repair in spite of loss of important domains. We reported previously [30] that Brh2 retains significant activity in DNA repair and homologous recombination despite deletion of the entire C-terminal region containing the canonical DNA-binding domain (Brh2ΔC551) or else swapping of that region for a heterologous DNA-binding domain (Brh2ΔC551::RPA70), namely the tandem OB-fold repeats from RPA70 (see Fig. 3A). Another structurally unrelated DNA-binding domain, the NBD, located in the N-terminal region of Brh2[31, 32] can apparently provide the necessary DNA-binding activity to enable Brh2 function in the absence of the canonical DNA binding domain. With the canonical binding domain present the NBD was found to be unnecessary for DNA repair proficiency except in the absence of Rad52 as evident by expression of Brh2Δ362-493 in *brh2*Δ *rad52*Δ (Fig. 3B) and [25, 27]. However, the requirement for Rad52 could be eliminated by substituting a heterologous DNA binding domain (tandem OB fold A and B modules from RPA70) for NBD (Brh2Δ362-493::OB-AB).

Regardless of their proficiency in DNA repair and activity in supporting homologous recombination, these Brh2 variants appeared to function differently under conditions of replication stress in producing intermediates that become toxic in the absence of Blm.

Presumed toxic DNA intermediates accumulated in *brh2*Δ *blm-KR* mutant cells expressing Brh2 or Brh2ΔC551::RPA70 as evident by the HU sensitivity of the strains expressing these Brh2 variants (Fig. 3C). Toxic DNA intermediates did not accumulate in *brh2*Δ *blm-KR* strains expressing any of the other variants as evident by their resistance to HU.

In view of the observation that Gen1, along with Blm, appears to serve in a common pathway dedicated to processing replication-stress associated toxic intermediates generated by Brh2-driven recombination, we interrogated the activity of the Brh2 variants in promoting sensitivity to HU (Fig. 3C). We reasoned that if *brh2*Δ *blm-KR* and *brh2*Δ *gen1*Δ were to respond in an equivalent manner to all the variants, this would support the contention that Blm and Gen1 participate in the same pathway. The results were unequivocal (Fig. 3C)–the Brh2 forms (Brh2 and Brh2ΔC551::RPA70) producing HU-induced toxic intermediates requiring Blm for processing showed the same requirement for Gen1, while the Brh2 variants that were less effective in promoting a toxic effect in the absence of Blm also were less effective in the absence of Gen1.

It should be noted, however, that Brh2 derivatives lacking one or the other DNA-binding domain (*i.e.*, Brh2ΔC551 or Brh2Δ362-493) were less effective in supporting DNA repair activity as measured by loss of resistance to UV when expressed in *brh2*Δ *gen1*Δmutant cells compared to *brh2*Δ cells (Fig. 3C). Note also that the requirement for Gen1 could be reduced by substituting a heterologous DNA binding for either N-terminal or C-terminal DNA-binding domain. In this regard the dependence of Brh2Δ362-493 on Gen1 for repair proficiency mirrored its dependence on Rad52 (Fig. 3B). Nevertheless, in promoting toxicity of *brh2*Δ *blm-KR* and *brh2*Δ *gen1*Δ mutant cells to HU only the chimeric variant Brh2ΔC551::RPA70 was effective. The other variant (Brh2Δ362- 493::OB-AB) followed the same pattern observed in *brh2*Δ *blm-KR* mutant cells in being less capable of inducing sensitivity.

The above results suggest that toxic intermediates accumulating during replication stress as a result of homologous recombination must be processed by both Blm and Gen1, but that some intermediates can be produced that are not toxic in the absence of either of these cellular factors. These intermediates, however, could presumably be processed by Mus81. Direct support for this notion would require testing of the quadruple mutant (*brh2Δ blm-KR mus81Δ gen1Δ*). Unfortunately, given that we have been unable to generate even *brh2Δ gen1Δ mus81Δ* triple mutants either by transformation or genetic crosses, the quadruple mutant is presumably also a synthetically lethal combination. Nevertheless, we were able to isolate a *brh2Δ blm-KR mus81Δ* mutant and so were interested to examine the response of this mutant to the Brh2 variants. However, only the Brh2 forms lacking one or the other DNA-binding domain could be relatively tolerated in the *brh2Δ blm-KR mus81Δ* background, Cells transformed with these variants grew robustly into colonies (Fig. 3D). The Brh2ΔC551::RPA70 hybrid that has previously been shown to be hyperrecombinogenic [30] exhibited an extreme toxicity —transformed cells were unable to grow into colonies. Under condition of replicative stress the Brh2ΔC551 and Brh2Δ362-493 variants were not effective in promoting toxicity in the absence of both Blm and Mus81 function (Fig. 3E). Furthermore, it is important to note that these two Brh2 variants were also less effective in supporting DNA repair, as measured by loss of resistance to UV, when expressed in *brh2Δ*

blm-KR mus81Δ mutant cells compared to *brh2*Δ *blm-KR* cells suggesting that these crippled Brh2 forms require Mus81 for competent DNA repair activity.

3.4 Resolvase dependence of Brh2 variants deleted of DNA-binding domains

The dependence of the Brh2Δ362-493 variant on Rad52 in complementing the UV sensitivity of *brh2*Δ initially raised the notion that there might be some commonality or functional overlap between the associated DNA-binding activities of the Brh2 NBD and Rad52. This view was reinforced by in vitro findings with purified proteins showing that the NBD could promote annealing of complementary single strands of DNA [32], an activity associated with Rad52 [33]. However, the dependence of Brh2Δ362-493 on Gen1 in complementing the UV of *brh2*Δ as noted here (Fig. 3C) suggested this notion was too simplistic.

To investigate this point further we tested whether the DNA repair functional response of the Brh2 variants deleted of the NBD or canonical DNA binding domain was dependent on Mus81 (Fig. 4A). As in the case of *brh2*Δ *gen1*Δ expression of Brh2Δ362-493 in the *brh2*Δ *mus81*Δ mutant was poorly effective in complementing the UV sensitivity (Fig. 4B). Furthermore, the Brh2Δ362-493::OB-AB variant was only partially effective in complementing UV sensitivity of *brh2*Δ *mus81*Δ compared to its activity in *brh2*Δ *rad52*Δ and *brh2*Δ *gen1*Δ (Fig. 3B,C). On the other hand expression of Brh2ΔC551 in *brh2*Δ *mus81*Δ restored UV resistance (Fig. 4B) in contrast to its limited activity in *brh2*Δ *gen1*Δ (Fig. 3C). These results suggest a different and perhaps more indispensable connection between Brh2 and Mus81 in their molecular interplay. In other words, the observation that Brh2Δ362-493 and Brh2Δ362-493::OB-AB are poorly effective in restoring the repair capacity of the *brh2*Δ *mus81*Δ mutant might point to a specific interplay between Brh2 and Mus81 rather than merely reflecting a requirement for resolvase activity in a broad sense.

To probe the possibility that recombination-intermediate resolvase activity could contribute to Brh2 functionality in DNA repair in the absence of the NBD, we tested whether a heterologous resolving activity could substitute. Prokaryotic Holliday junction resolvase RusA was able to restore UV resistance to both *blm-KR mus81*Δ and *gen1*Δ *mus81*Δ (Fig. 4C) suggesting RusA is able to compensate for the absence of Mus81. However, RusA failed to restore DNA repair activity to *brh2*Δ *mus81*Δ expressing Brh2Δ362-493 (Fig. 4D). This finding indicates that DNA repair proficiency of Brh2 lacking the NBD is more likely dependent on cognate functions of Mus81. One final point is that the Brh2 variants effective in promoting toxicity under replication stress in *brh2*Δ *mus81*Δ were the same that were effective in *brh2Δ blm-KR* and *brh2Δ gen1Δ* (compare Fig. 4B with Fig. 3C). This is reason to believe that these three cellular factors contributing to management of potentially toxic recombination intermediate DNA structures are involved in a common pathway.

4. Discussion

Processing DNA after damage is necessary to prepare it as a substrate for the homologous recombination machinery, the advent of which is brought into play by the association of Brh2. Additional DNA processing is necessary after the action of Brh2 and its effector Rad51 to resolve newly formed structural intermediates. It would be incorrect to picture the

resolution process as a reversal of the preparative stage, but nonetheless it is interesting to consider that one well-characterized processing factor, namely Blm, serves in both the preparative and resolving or dissolution phases–its helicase activity being harnessed in different complexes for alternative purposes. This investigation was prompted by (i.) curiosity about possible roles of Mus81 and Gen1 in DNA processing at steps other than the resolution phase as envisioned by the current dogma, and (ii.) the possibility of interplay between components of the homologous recombination and resolution systems.

Two main conclusions can be drawn from this work. First, in *U. maydis* homologous recombination-intermediate-resolving factors that contribute to repair of damage by UV or MMS and that resolve toxic DNA structures resulting from HU poisoning during replication are partially redundant. Gen1 can compensate to a certain extent for loss of Blm function in countering replication stress caused by HU poisoning but only in the absence of Mus81. Like Blm, Gen1 is also required to eliminate HU-induced toxic intermediates formed by the homologous recombination pathway. Second, repair proficiency of Brh2 mutant variants deleted of its N-terminal DNA binding domain requires not only Rad52 but also Gen1 and Mus81. This suggests the operation of a Rad52-dependent/Gen1-Mus81-dependent system for protection of genome integrity that is ordinarily not evident, but which is revealed when the primary Rad51 mediator, namely Brh2, is compromised in DNA interaction. We think of this system as a below-the-surface or subterranean repair shunt. A heterologous recombination-intermediate resolvase can substitute for endogenous resolution activities in repair of UV damage, but is not effective in repair by the subterranean system.

4.1 Contributions of Mus81 and Gen1 in resolving replication-stress- associated structures

Studies on the regulation of the yeast and human resolvases in the cell cycle by control of phosphorylation state and nuclear localization have led to a model in which there are consecutive waves of resolution action at metaphase and anaphase [9]. Subsequent to the decatenation and unlinking of recombination intermediates by the Sgs1/BLM helicase in concert with Top3/TOPIIIa early in the cell cycle, current dogma features activation of Yen1/GEN1 following the action of Mus81 in resolving any uncut recombination intermediates linking sister chromatids as a final means for ensuring their segregation.

The model envisioned above is adapted to processing of double Holliday junction recombination intermediates formed after introduction of double strand breaks. However, it is not clear what types of DNA intermediate structures form in response to damage by UV or MMS, which generates lesions that interfere with replication, or in response to poisoning by HU, which causes replication fork stalling. It is notable that *U. maydis* cells lacking either Blm or Gen1 function are resistant to UV and MMS damage, but are sensitive to HU. It is likely that Mus81 can compensate for the absence of Blm or Gen1 in processing intermediates induced by UV as the double mutant combinations are sensitive. In fact, the finding that the *blm-KR mus81*Δ mutant was highly sensitive to UV and MMS while *blm-KR gen1*Δ was only mildly sensitive underscores the primacy of Mus81 among these proteins in combating the genotoxic effects of UV and MMS.

Inactivating homologous recombination in Blm- or Gen1-deficient cells restores HU resistance, suggesting that whatever type of toxic intermediate structures induced by HU

requires Brh2 for formation and both Blm and Gen1 for removal. From the above data, the markedly similar response of *blm-KR* and *gen1*Δ mutants could most simply be accounted for by cooperation of the two gene products in the same pathway. The epistatic relationship evident by the *blm-KR gen1*Δ double mutant reinforces this notion and further suggests the possibility not only that Gen1 acts in the same pathway with Blm but somehow upstream of Blm. Given that the *blm-KR gen1*Δ double mutant is more resistant to HU than the *blm-KR* single mutant it seems less likely that Gen1 acts downstream of Blm. Of course, these considerations do not rule out the possibility that Blm and Gen1 could operate alternately in multiple steps throughout the course of the processing of the recombination intermediates with a demand for a final involvement of Blm. If true, this scenario could account for the finding that the *blm-KR gen1*Δ double mutant was marginally more resistant to HU than either single mutant.

On the other hand, expressing Gen1 constitutively abrogates the need for Blm in countering HU poisoning, but not UV or MMS damage. However, this can happen only if *blm-KR* cells lack Mus81. This finding is of special note because it is most suggestive of the possibility for the ordered and directed actions of Mus81, Gen1, and Blm in the processing of toxic DNA structures arising from replication stress. It would appear that because the *blm-KR* cells contain Mus81 the aberrant DNA structures are rendered for further processing by Blm and Gen1. Furthermore, the observation that the suppression by Gen1 is completely eliminated when Mus81 is present could reflect a hierarchical ordering in resolvase deployment such that in the absence of Blm, Mus81 becomes extremely competitive for the perturbed-replication-fork structures. Two additional observations bearing on the issue of mitigating the toxicity of the replication-associated recombination intermediates deserve mention in this context. Although *mus81*Δ is less sensitive to HU, disabling homologous recombination in Mus81 deficient cells restores HU resistance indicating that, like Blm and Gen1, Mus81 is involved in removal of the Brh2-induced recombination intermediates. Moreover, the cells lacking any of these three cellular factors respond equivalently to the Brh2 variants making it logical to suppose that they are involved in common or analogous function. A model featuring relationships among these above factors and their hierarchical arrangements as described is depicted in a schematic (Fig. 5). We imagine multiple points of interaction as intermediates are processed.

There is, however, a caveat to the above model that might warrant a reinterpretation of the findings. The strain carrying the *blm-KR* allele used in these studies was chosen for experimentation because it does not harbor a drug resistance marker. This provides an extra degree of freedom for making additional gene disruptions in this strain because the number of drug resistance markers available in *U. maydis* is limited. The functionally inactive Blm-KR gene product, however, could be acting in a dominant negative fashion that biases resolution. To wit, after HU induced fork arrest, the HR machinery produces a potentially toxic intermediate, which under the normal conditions would be dissolved by Blm helicase. But in the *blm-KR* mutant strain the Blm-KR variant could be attracted to the toxic intermediate only to serve as an obstacle for other alternative helicases and as a consequence force resolution by the resolvases. Indeed, the preference of Gen1 over Mus81 could be related to the specific structure of the Blm-KR-DNA intermediate that demands a symmetric resolution as would be provided by Gen1 versus an asymmetric resolution as would be

provided by Mus81. This might explain why the ability of Gen1 to rescue the hydroxyurea sensitivity of *blm-KR* requires the absence of Mus81. If the preferred resolvase was Mus81 and its asymmetric resolution of the DNA intermediate resulted in a toxic structure, then only the absence of Mus81 might allow Gen1 to do the work properly. These issues will require further experimentation. We note, however, regarding the first point of Blm-KR forcing resolution by resolvases that deletion of Gen1 improves survival of the *blm-KR* strain; and regarding the second point of toxic intermediate production by Mus81 that the *blm-KR gen1*Δ mutant is more resistant to HU than *blm-KR mus81*Δ. These observations are not consistent with this scenario of a dominant negative Blm.

4.2 Impact of Rad51 mediator status on resolution mode

The other finding in this study of particular interest is that repair proficiency of Brh2 mutant variants deleted of the NBD requires not only Rad52 but also Gen1 and Mus81. Brh2 in its role as mediator of Rad51 is capable of providing DNA repair activity and supporting homologous recombination even in the absence of one of its two different DNA-binding regions. However, the requirements are different. When the NBD is deleted, repair and recombination activity as gauged by resistance to UV is dependent on functional Rad52. But when the canonical C-terminal DNA binding domain is removed recombinational repair is Rad52 independent. In contrast, strains expressing either deletion variant require Gen1 for repair proficiency. However, *gen1*Δ strains expressing these variants do not succumb to HU poisoning, unlike *gen1*Δ strains expressing the natural Brh2 or Brh2ΔC551::RPA70 chimera. These findings reinforce the notion that an appropriate architectural configuration of DNA-binding domains within Brh2 dictates the type of structural DNA intermediate to form during repair. The nature of the intermediate, in turn, dictates the choice of factors necessary for processing and resolution by both the canonical and subterranean pathways of homologous recombination. The challenge in understanding molecular events performed by Brh2 during repair of damage and restoration of replication fork progression after stress is to define the DNA structures formed during these processes. Understanding the genetic and molecular events will provide new insights into elaborate system that serves to advance DNA replication and preserve the integrity of the genome.

To summarize, we suggest that cellular factors involved in recombination and resolution of DNA intermediate structures form an ensemble of components whose interplay creates a stream of alternate participatory actions that executes well-oiled processing of the damaged or perturbed DNA structure. In other words, the ensemble operates in a way that lets the recombination enablers and resolvers collaborate in a spectrum of operations. The extent and direction of this collaboration are ordered by the demand for specific steps in repair that are in turn dictated by the type of structural perturbation. In this scenario, Brh2 must cooperate with the structure-resolving functions to achieve well orchestrated DNA repair. From the results described above, it seemed logical to propose this model of collaboration in the actions of Brh2 by resolving factors during DNA repair and replication stress, but the molecular events underlying this cooperation and the molecular structures of the DNA intermediates formed during the process are challenging mechanistic issues awaiting insightful experimentation.

Acknowledgments

This work received support from National Institutes of Health grant GM079859 (WKH) and from grant 173005 from the Ministry of Education, Science and Technology, Republic of Serbia. We thank Ninghui Mao (WKH laboratory) for strain construction. We thank Lorraine Symington (Columbia University) and Jeanette Sutherland (WKH laboratory) for discussion and comments on the manuscript. We thank an astute reviewer for pointing out an alternative interpretation of some of the data.

References

1. Gaillard H, Garcia-Muse T, Aguilera A. Replication stress and cancer. Nat Rev Cancer. 2015; 15:276–289. [PubMed: 25907220]
2. Magdalou I, Lopez BS, Pasero P, Lambert SA. The causes of replication stress and their consequences on genome stability and cell fate. Semin Cell Dev Biol. 2014; 30:154–164. [PubMed: 24818779]
3. Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nat Cell Biol. 2014; 16:2–9. [PubMed: 24366029]
4. Berti M, Vindigni A. Replication stress: getting back on track. Nat Struct Mol Biol. 2016; 23:103–109. [PubMed: 26840898]
5. Branzei D, Szakal B. DNA damage tolerance by recombination: Molecular pathways and DNA structures. DNA Repair (Amst). 2016; 44:68–75. [PubMed: 27236213]
6. Blanco MG, Matos J. Hold your horSSEs: controlling structure-selective endonucleases MUS81 and Yen1/GEN1. Front Genet. 2015; 6:253. [PubMed: 26284109]
7. Matos J, West SC. Holliday junction resolution: regulation in space and time. DNA Repair (Amst). 2014; 19:176–181. [PubMed: 24767945]
8. Rass U. Resolving branched DNA intermediates with structure-specific nucleases during replication in eukaryotes. Chromosoma. 2013; 122:499–515. [PubMed: 24008669]
9. West SC, Blanco MG, Chan YW, Matos J, Sarbajna S, Wyatt HD. Resolution of Recombination Intermediates: Mechanisms and Regulation. Cold Spring Harb Symp Quant Biol. 2015; 80:103–109. [PubMed: 26370409]
10. Fabre F, Chan A, Heyer WD, Gangloff S. Alternate pathways involving Sgs1/Top3, Mus81/ Mms4, and Srs2 prevent formation of toxic recombination intermediates from single-stranded gaps created by DNA replication. Proc Natl Acad Sci U S A. 2002; 99:16887–16892. [PubMed: 12475932]
11. Bastin-Shanower SA, Fricke WM, Mullen JR, Brill SJ. The mechanism of Mus81-Mms4 cleavage site selection distinguishes it from the homologous endonuclease Rad1-Rad10. Mol Cell Biol. 2003; 23:3487–3496. [PubMed: 12724407]
12. Cejka P, Plank JL, Bachrati CZ, Hickson ID, Kowalczykowski SC. Rmi1 stimulates decatenation of double Holliday junctions during dissolution by Sgs1-Top3. Nat Struct Mol Biol. 2010; 17:1377–1382. [PubMed: 20935631]
13. Ira G, Malkova A, Liberi G, Foiani M, Haber JE. Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast. Cell. 2003; 115:401–411. [PubMed: 14622595]
14. Wu L, Hickson ID. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature. 2003; 426:870–874. [PubMed: 14685245]
15. Kaliraman V, Mullen JR, Fricke WM, Bastin-Shanower SA, Brill SJ. Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease. Genes Dev. 2001; 15:2730–2740. [PubMed: 11641278]
16. Agmon N, Yovel M, Harari Y, Liefshitz B, Kupiec M. The role of Holliday junction resolvases in the repair of spontaneous and induced DNA damage. Nucleic Acids Res. 2011; 39:7009–7019. [PubMed: 21609961]
17. Ho CK, Mazon G, Lam AF, Symington LS. Mus81 and Yen1 promote reciprocal exchange during mitotic recombination to maintain genome integrity in budding yeast. Mol Cell. 2010; 40:988–1000. [PubMed: 21172663]
18. Tay YD, Wu L. Overlapping roles for Yen1 and Mus81 in cellular Holliday junction processing. J Biol Chem. 2010; 285:11427–11432. [PubMed: 20178992]

19. Blanco MG, Matos J, Rass U, Ip SC, West SC. Functional overlap between the structure-specific nucleases Yen1 and Mus81-Mms4 for DNA-damage repair in *S. cerevisiae*. DNA Repair (Amst). 2010; 9:394–402. [PubMed: 20106725]
20. Boddy MN, Lopez-Girona A, Shanahan P, Interthal H, Heyer WD, Russell P. Damage tolerance protein Mus81 associates with the FHA1 domain of checkpoint kinase Cds1. Mol Cell Biol. 2000; 20:8758–8766. [PubMed: 11073977]
21. Andersen SL, Kuo HK, Savukoski D, Brodsky MH, Sekelsky J. Three structure-selective endonucleases are essential in the absence of BLM helicase in Drosophila. PLoS Genet. 2011; 7:e1002315. [PubMed: 22022278]
22. Saito TT, Mohideen F, Meyer K, Harper JW, Colaiacovo MP. SLX-1 is required for maintaining genomic integrity and promoting meiotic noncrossovers in the *Caenorhabditis elegans* germline. PLoS Genet. 2012; 8:e1002888. [PubMed: 22927825]
23. Kojic M, Kostrub CF, Buchman AR, Holloman WK. BRCA2 homolog required for proficiency in DNA repair, recombination, and genome stability in *Ustilago maydis*. Mol Cell. 2002; 10:683–691. [PubMed: 12408834]
24. Mao N, Kojic M, Holloman WK. Role of Blm and collaborating factors in recombination and survival following replication stress in *Ustilago maydis*. DNA Repair (Amst). 2009; 8:752–759. [PubMed: 19349216]
25. Kojic M, Holloman WK. Brh2 domain function distinguished by differential cellular responses to DNA damage and replication stress. Mol Microbiol. 2012; 83:351–361. [PubMed: 22171788]
26. Kojic M, Mao N, Zhou Q, Lisby M, Holloman WK. Compensatory role for Rad52 during recombinational repair in *Ustilago maydis*. Mol Microbiol. 2008; 67:1156–1168. [PubMed: 18208529]
27. Kojic M, Zhou Q, Fan J, Holloman WK. Mutational analysis of Brh2 reveals requirements for compensating mediator functions. Mol Microbiol. 2011; 79:180–191. [PubMed: 21166902]
28. Interthal H, Heyer WD. MUS81 encodes a novel helix-hairpin-helix protein involved in the response to UV- and methylation-induced DNA damage in *Saccharomyces cerevisiae*. Mol Gen Genet. 2000; 263:812–827. [PubMed: 10905349]
29. Lorenz A, West SC, Whitby MC. The human Holliday junction resolvase GEN1 rescues the meiotic phenotype of a *Schizosaccharomyces pombe mus81* mutant. Nucleic Acids Res. 2010; 38:1866–1873. [PubMed: 20040574]
30. Kojic M, Zhou Q, Lisby M, Holloman WK. Brh2-Dss1 interplay enables properly controlled recombination in *Ustilago maydis*. Mol Cell Biol. 2005; 25:2547–2557. [PubMed: 15767662]
31. Zhou Q, Kojic M, Holloman WK. DNA-binding Domain within the Brh2 N Terminus Is the Primary Interaction Site for Association with DNA. J Biol Chem. 2009; 284:8265–8273. [PubMed: 19182269]
32. Zhou Q, Holloman WK. Dual DNA-binding domains shape the interaction of Brh2 with DNA. DNA Repair (Amst). 2014; 22:104–111. [PubMed: 25128760]
33. Mazloum N, Zhou Q, Holloman WK. DNA binding, annealing, and strand exchange activities of Brh2 protein from *Ustilago maydis*. Biochemistry. 2007; 46:7163–7173. [PubMed: 17523678]

Highlights

- Gen1 can rescue hydroxyurea sensitivity of mutant cells with dysfunctional Blm only in the absence of Mus81.
- Mutants defective in Blm and Gen1 respond similarly to hydroxyurea challenge.
- Brh2 mutant form deleted of its N-terminal DNA binding region requires the presence of Gen1 and Mus81 for DNA repair proficiency.

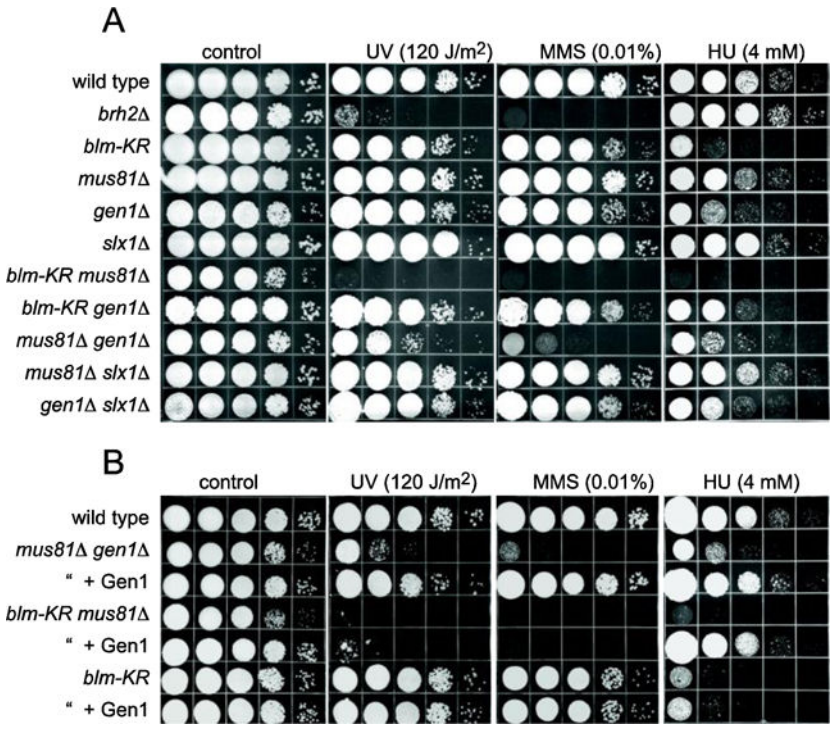

Figure 1.

Role of resolvases in survival after DNA damage or replication stress. A. Mutant strains were tested for sensitivity to UV, MMS and HU as described in Experimental procedures. B. Mutant strains expressing Gen1 from a self-replicating plasmid (indicated as + Gen1) were tested for sensitivity. Results shown here and in subsequent figures are representative of multiple determinations. At least three independent isolates and measurements were performed for each strain.

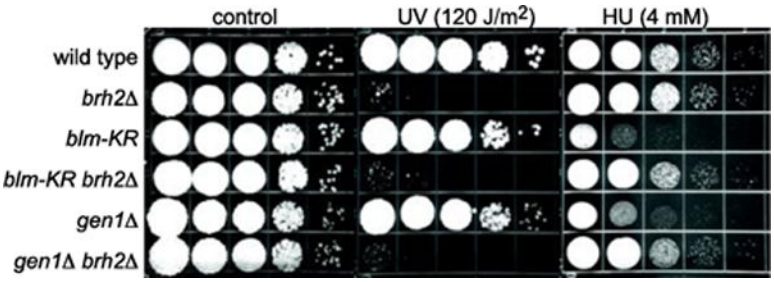

Figure 2.

Suppression of HU sensitivity by inactivation of homologous recombination. Single and double mutant combinations were tested for sensitivity to UV and HU.

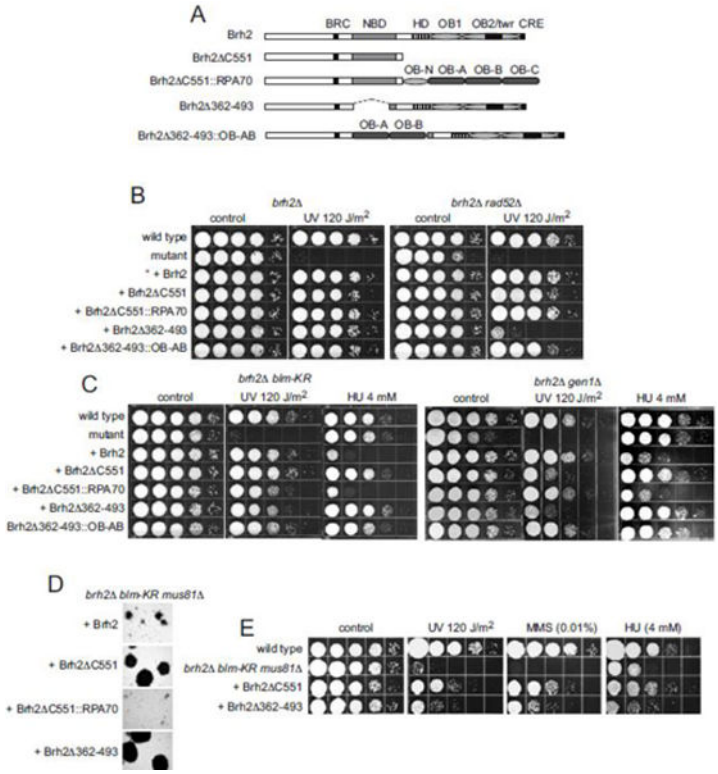

Figure 3.

DNA-binding domain dependence of Brh2 in supporting DNA repair and suppressing HU poisoning. A. Schematic representation of Brh2 variants with deletions or substitutions of DNA-binding domains. Structural motifs and functional regions of Brh2 are denoted in order: BRC, Rad51-interaction motif; NBD, N-terminal DNA binding region; HD, helix-rich domain; OB1 and OB2/twr, OB folds and intervening tower domain; CRE, C-terminal Rad51 interaction element. OB fold modules of RPA70 are also indicated. B. DNA repair activity of strains expressing the Brh2 variants. C. DNA repair and replication stress resistance of strains expressing the Brh2 variants. D. Micrographs of emerging microcolonies viewed with 40x objective after simultaneous transformation to of *brh2*Δ *blm-KR mus81*Δ cells with plasmids expressing the indicated Brh2 variants. Plasmid carried a gene expressing resistance to carboxin for selection. E. DNA repair and replication stress of strains expressing the Brh2 variants.

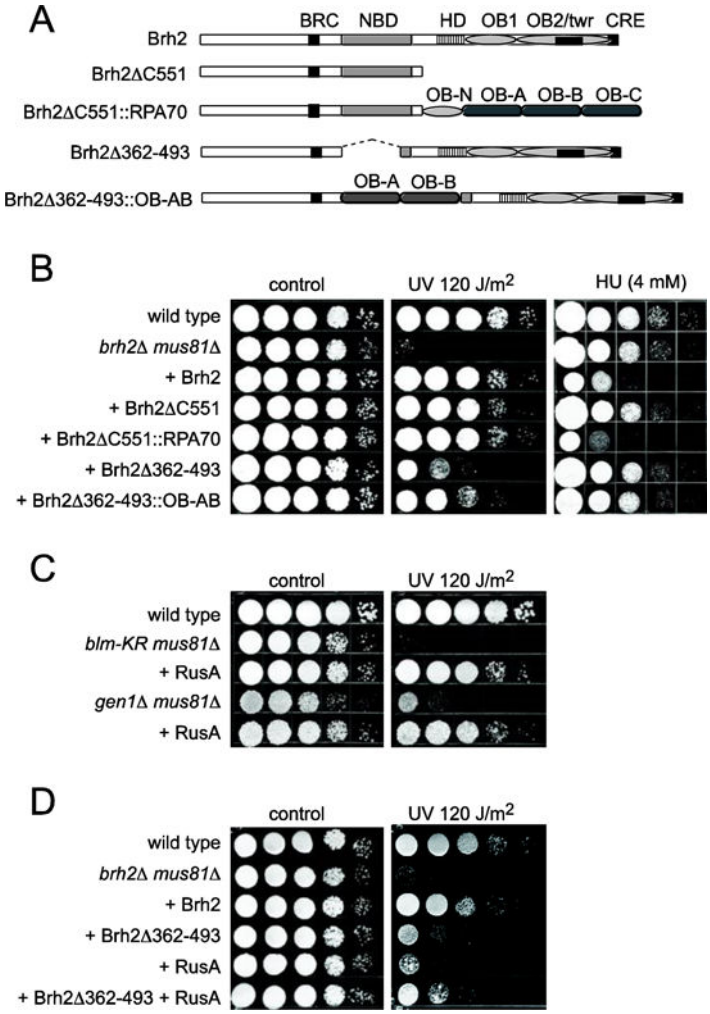

Figure 4.

Brh2 and RusA dependence in supporting DNA repair in the absence of Mus81. A. Schematic of Brh2 deletion or hybrid variants. B. Survival of double mutants expressing Brh2 variants. C. Survival of double mutants expressing RusA. D. Survival of double mutant expressing RusA or Brh2 or Brh2 deleted of NBD.

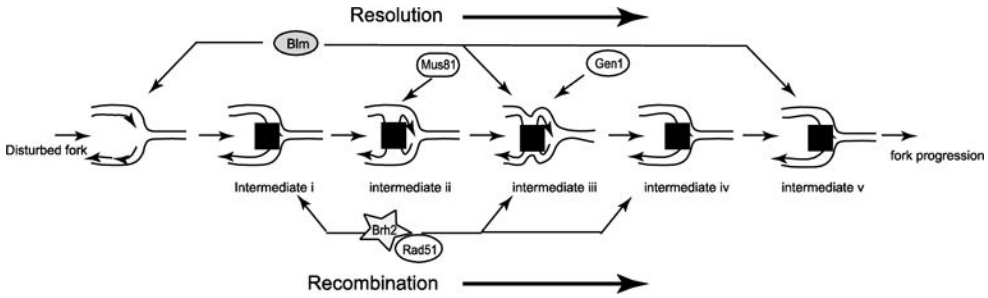

Figure 5.

Collaboration among resolving factors in processing structural intermediates formed after replication stress. The hypothetical model illustrates multiple points of interaction of resolving factors Blm, Mus81, and Gen1 in formation and resolution of intermediates formed by Brh2/Rad51 after replication stress. It is imagined that Brh2/Rad51 action can result in different types of intermediates and that the factors shown can intervene at different steps in the pathway leading to restoration of fork progression. The black boxes represent different recombination intermediates of unknown structures.