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1 **Homologous recombination and Mus81 promote replication**
2 **completion in response to replication fork blockage**

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21

1 **ABSTRACT**

2

3 Impediments to DNA replication threaten genome stability. The homologous
4 recombination (HR) pathway has been involved in the restart of blocked replication forks.
5 Here, we used a method to increase yeast cell permeability in order to study at the molecular
6 level the fate of replication forks blocked by DNA topoisomerase I poisoning by
7 camptothecin (CPT). Our results indicate that Rad52 and Rad51 HR factors are required to
8 complete DNA replication in response to CPT. Recombination events occurring during S
9 phase do not generally lead to the restart of DNA synthesis but rather protect blocked forks
10 until they merge with convergent forks. This fusion generates structures requiring their
11 resolution by the Mus81 endonuclease in G₂/M. At the global genome level, the multiplicity
12 of replication origins in eukaryotic genomes and the fork protection mechanism provided by
13 HR appear therefore to be essential to complete DNA replication in response to fork
14 blockage.

1 INTRODUCTION

2

3 Chromosome duplication during the S phase is a crucial step of cell division. DNA
4 replication in eukaryotes is initiated from multiple origins distributed on all chromosomes.
5 Replication forks progress along chromosomes arms until merging with another incoming
6 fork at termination regions. The progression of replication forks can be hindered by obstacles
7 in the DNA template such as DNA lesions. Genome stability is particularly at risk when
8 damaged DNA molecules are replicated. Failure in DNA damage repair can lead to the
9 terminal arrest or breakage of replication forks, and ultimately to the distribution of under-
10 replicated and / or broken chromosomes to the daughter cells after mitotic division. When a
11 fork becomes dysfunctional, the completion of replication could be ensured by a converging
12 functional fork, a process that can be favored by the firing of nearby dormant origins [1,2].
13 Alternatively, dysfunctional replication forks could also be restarted, a mechanism that
14 requires the homologous recombination (HR) pathway [3-5]. During HR, Rad52 and Rad51
15 are fundamental to coat the ssDNA generated by resection and carry out the strand invasion
16 and exchange reactions [6]. At dysfunctional forks, Rad51 loading by Rad52 also regulates
17 nascent DNA strands degradation by exonucleases [7-9]. Whether this protection mechanism
18 is different from the restart mechanism has not been clearly determined [7,10-12].

19 Recombination-mediated replication restart has been mainly studied in the yeast
20 cellular model using site-specific tools to break or block replication forks at a unique location
21 [3,4,13-17]. Replication restart in *Saccharomyces cerevisiae* has been recently investigated
22 using a Flp-nick system, which produces a site-specific DNA nick that is converted into a
23 one-ended DSB upon the passage of a replication fork [4,14,18]. Recombination-mediated
24 restart seems to occur by break-induced replication (BIR), an HR pathway mainly
25 characterized outside of S phase [19,20]. BIR is favored when only one DSB end is able to
26 invade a homologous template. In that case, DNA synthesis primed within the displacement
27 loop (D-loop) can proceed to the chromosome end by a conservative mechanism [21-24]. In
28 particular, the non-essential DNA polymerase δ subunit Pol32 is required for priming DNA
29 synthesis inside the D-loop and the Pif1 DNA helicase is required to promote its migration
30 [24-26]. Lastly, BIR synthesis is highly mutagenic [4,27] and subjected to template switching,
31 leading to complex chromosomal rearrangements [28-31]. Terminally-blocked replication
32 forks at the *RTSI* barrier in *Schizosaccharomyces pombe* can be restarted by HR without DSB
33 formation [3,7,32-35]. Similar to BIR in *S. cerevisiae*, replication restart at *RTSI* requires the
34 DNA polymerase δ and the Pfh1 DNA helicase, is highly mutagenic and prone to template

1 switching [35-38]. In that context, HR is thought not to be primed from a DSB but from the
2 tip of a reversed fork, which results from the displacement and reannealing of the nascent
3 strands together [32,39,40].

4 It remains to be determined if replication restart studied at locus-specific barriers
5 occurs in the same way at the genome-wide level in response to natural replication
6 impediments. For example, the DNA topoisomerase 1 (Top1) normally introduces a transient
7 nick to relax supercoiled DNA during transcription and replication [41]. During this reaction,
8 Top1 remains covalently attached to the 3' end of the break, forming a "cleavage complex"
9 (Top1cc), before the relaxation of DNA and religation of the break. Cells are constantly
10 challenged with blocked Top1ccs, which is a major driver of mutagenesis in highly
11 transcribed genes [42,43]. Furthermore, cells devoid of both Tdp1 and Wss1, two factors
12 involved in the removal of Top1ccs, have a severe Top1-dependent growth defect [44,45],
13 which indicates that Top1ccs are natural threats to cell survival. Top1ccs can also be
14 stabilized by the drug camptothecin (CPT) [46]. CPT is thought to cause the formation of a
15 one-ended DSB, the typical substrate for BIR, upon the collision of the replication fork with
16 the nick in the Top1cc, thus inducing a replication stress [47-49]. However, CPT-induced
17 DSBs have not been observed in yeast cells [50,51]. More recently, it has been shown that
18 CPT treatment of yeast cells induces fork reversal [50,52], as a possible consequence of
19 accumulation of positive supercoils ahead of replication forks due to Top1 inhibition [53].
20 Hence, replication fork blockage by CPT-stabilized Top1ccs appears as a relevant model to
21 address if replication restart occurs at the global genomic level.

22 Finally, it remains unclear which factors participate in the resolution of the
23 recombination intermediates during HR-mediated restart of DNA replication. The structure-
24 selective endonuclease Mus81 has been proposed to cleave either blocked or reversed forks to
25 promote repair by HR [54-56]. However, Mus81 is not required for replication restart at *RTS1*
26 in fission yeast, nor for the repair of a replication-born DSB in budding yeast [3,17]. Mus81 is
27 nevertheless involved in the processing of these recombination events, as they accumulate in
28 its absence [3,17], resulting in a decreased amount of final repair products [17,57]. By using
29 the Flp-nick system, it has been proposed that Mus81 limits replication restart by Pol32-
30 dependent BIR in S phase by processing the migrating D-loop [4]. Mus81 catalytic activity is
31 normally very low in S phase, and only increases at the G₂/M transition, when the Mms4
32 regulating subunit of the complex is hyper-phosphorylated by multiple kinases [58-61].
33 Hence, the role of Mus81 in replication restart by HR in S phase appears contradictory to the

1 regulation of its activity. We took advantage of our study to clarify the role of Mus81 in
2 recombination-mediated restart of DNA replication.

3

4 Our data reveal that HR is primarily providing a replication fork protection
5 mechanism rather than promoting the restart of DNA synthesis from blocked forks. Thanks to
6 a method to increase CPT entry into yeast cells, we show that S phase completion in response
7 to Top1 poisoning requires Rad52 and Rad51. We propose that HR promotes the formation of
8 a D-loop structure that protects blocked forks until they merge with converging forks.
9 Completion of replication also requires Mus81 in G₂/M to promote the termination of DNA
10 replication at protected forks. We confirmed our results in CPT-treated cells using an
11 independent system in which a specific mutation of the Rad3 DNA helicase generates a
12 similar replication stress. This allowed us to demonstrate that the mechanism we have
13 characterized upon Top1 poisoning is independent of the accumulation of supercoiled DNA
14 and DNA-protein crosslinks. The engagement of HR to protect blocked forks and to ensure
15 the completion of DNA replication appears therefore as a general mechanism to respond to
16 replication stress.

1 RESULTS

2

3 **Top1 poisoning by CPT and the *rad3-102* allele exert a genetically similar replication** 4 **stress**

5 In this study, we have chosen to study the dynamics of DNA replication challenged by
6 CPT treatment, which exacerbates at the genome-wide level the presence of trapped Top1 on
7 the DNA template. Resistance to CPT-induced DNA damage absolutely requires the
8 homologous recombination (HR) machinery, as null mutations of *RAD52*, *MRE11*, *RAD50*
9 and *XRS2* lead to the highest sensitivities to low doses of CPT compared to wild type cells
10 (**Figure 1A**) [62,63]. *rad51Δ* mutants are also highly sensitive to low CPT doses but to a
11 lesser extent than its upstream regulator Rad52 (**Fig 1B and C, Appendix Fig S1A**) [4].
12 Interestingly, Rad52 and the members of the MRX complex (Mre11-Rad50-Xrs2), but not
13 Rad51, are essential for the survival of cells bearing the *rad3-102* mutation: this mutation
14 impairs the nucleotide excision repair (NER) pathway by increasing the binding of the TFIIH
15 complex to a single-strand DNA gap intermediate, which prevents its subsequent filling
16 [64,65]. Additionally, it has been described that the elevated CPT sensitivity of a nuclease-
17 deficient Mre11 mutant (*mre11-3*) can be suppressed by the absence of the Ku complex and
18 in an Exo1-dependent manner (**Appendix Fig S1B**) [66]. This suggests that Mre11
19 counteracts the action of Ku at CPT-induced DSBs ends, which limits Exo1-dependent DNA
20 resection. Similarly, although the combination of *mre11-3* with *rad3-102* is not lethal, it
21 sensitizes the cells to UV exposure, a context in which the load of replication stress is
22 enhanced in the *rad3-102* background [65] (**Fig 1D**). Strikingly, the increased UV sensitivity
23 of *rad3-102 mre11-3* compared to *rad3-102* can be **partially** suppressed by the absence of Ku
24 in an Exo1-dependent manner (**Fig 1D and Appendix Fig S1C**). These data show that
25 Mre11, Ku and Exo1 play similar roles in DNA damage repair induced by *rad3-102* or CPT.
26 Finally, *rad3-102* has been found lethal in combination with both *rad51Δ* and *pol32Δ*, leading
27 to the proposal that DNA repair in the absence of Rad51 in these cells is backed up by the
28 presence of the Pol32 non-essential subunit of the DNA polymerase δ [65,67]. Remarkably,
29 we found that the *rad51Δ pol32Δ* double mutant was more sensitive to CPT than the *rad51Δ*
30 single mutant (**Fig 1B and C**), suggesting that, as in *rad3-102* cells, Pol32 also partially
31 compensates the absence of Rad51 to cope with CPT-induced DNA damage.

32 Thus, *rad3-102* cells suffer from a replication stress that mimics the effect of CPT.
33 However, accumulation of topological stress is not expected in *rad3-102* cells, since they are
34 not affected in DNA supercoils removal by Top1. Tdp1 (Tyrosyl-DNA phosphodiesterase 1),

1 nor the metalloprotease Wss1, are required for survival in *rad3-102* cells, consistent with the
2 TFIIH complex not being covalently linked to DNA (**Appendix Fig S1D**). Overall, in view of
3 the strikingly similar genetic requirements for the cell survival in response to CPT and in the
4 *rad3-102* background, we decided to use these two systems to further investigate the repair
5 mechanisms required to cope with this type of replication stress, independently from the
6 accumulation of DNA supercoiling and DNA-protein crosslinks.

7 8 **Rad51 and Pol32 independently promote DSB repair by break-induced replication**

9 First, we asked what could be the specific contribution of Pol32 to DNA repair in the
10 absence of Rad51. We previously proposed that Pol32 may stabilize strand invasion in the
11 absence of Rad51 by promoting the priming of DNA synthesis [67], further supported by the
12 essential role of Pol32 for viability of cells lacking Rad51 that are defective in histone H3K56
13 deacetylation [68]. Among DSB repair pathways mediated by HR, Pol32 has only an essential
14 role during BIR. In the absence of Pol32, the initiation of DNA synthesis during BIR is
15 compromised [25]. Moreover, the recovery of both Rad51-dependent and Rad51-independent
16 survivors in cells lacking telomerase, thought to occur by BIR, also rely on Pol32 [25].
17 Finally, the BIR pathway has been proposed to be involved in the repair of broken replication
18 forks [4]. We thus wondered if Pol32 compensatory function in the absence of Rad51 in cells
19 exposed to CPT or in the *rad3-102* background could be related to its function in BIR. To
20 assess the redundant role of Pol32 over Rad51 in BIR, we used a well-described chromosomal
21 system [23] in which a single DSB is induced by the HO endonuclease. In this system, only
22 one of the two ends can undergo homology-dependent strand invasion at an ectopic location.
23 Subsequent priming and elongation of DNA synthesis reaching the chromosome end leads to
24 the production of viable Lys²⁺ recombinants (**Fig 1E**). In this system, the absence of Rad52
25 decreased the BIR frequency by about three orders of magnitude compared to the wild type
26 (**Fig 1F**). Deletion of *RAD51* or *POL32* also significantly decreased the BIR frequency
27 compared to the wild type (**Fig 1F**) [23]. However, only the combined absence of Rad51 and
28 Pol32 did affect the BIR frequency as much as in the absence of Rad52 (**Fig 1F**). These
29 results show that Pol32 can promote BIR in the absence of Rad51 and indicate that Pol32
30 functions in BIR to stimulate both strand invasion and priming of extensive DNA synthesis.
31 The absence of Pol32 alone affects cell survival only in response to high CPT concentrations
32 (**Figure 1B**) [69]. Altogether, these results suggest Pol32-dependent BIR-mediated synthesis
33 is not the main pathway required to cope with CPT-induced DNA damage and that the role of

1 Pol32 in the absence of Rad51 may be ascribed to its function in stimulating strand invasion
2 during HR.

4 **Mus81 is involved in HR-mediated repair involving both Rad51 and Pol32**

5 In human cells, the structure-specific endonuclease (SSE) Mus81 has been shown to
6 generate DSBs in response to CPT treatment, leading to the suggestion that Mus81 may
7 cleave stalled or reversed replication forks upon Top1 poisoning to promote fork restart [54].
8 In budding yeast, null and catalytically dead (*mus81-dd*) mutations of *MUS81* have been
9 shown to sensitize cells to mild CPT doses (**Fig 2A**) [62,63,70-72]. The absence of Mus81 is
10 backed up by the Yen1 SSE, as shown by the higher CPT sensitivity of the double *mus81Δ*
11 *yen1Δ* mutant compared to *mus81Δ* (**Fig EV1A**) [71,72]. Moreover, Mus81 and Yen1 have
12 been proposed to limit BIR synthesis initiated from a replication-born DSB [4].

13 We asked if Mus81 could have a similar role in CPT-induced DNA damage repair.
14 First, we observed that the combination of *mus81Δ* with *rad51Δ* and *pol32Δ* mutations
15 resulted in an increased CPT sensitivity than either single mutants (**Fig 2B**) [4], suggesting
16 that Mus81 participates in both Rad51- and Pol32-dependent repair. As for *rad51Δ*, *mus81Δ*
17 or *mus81-dd* are not lethal in combination with *rad3-102* but the double mutant cells
18 exhibited a much higher sensitivity to UV than single mutant cells (**Fig 2C**) [65]. We
19 analyzed the redundancy between Mus81 and Yen1 for survival in the *rad3-102* background.
20 *rad3-102 yen1Δ* cells were not more sensitive to UV than *rad3-102* cells (**Fig EV1B**),
21 consistent with *yen1Δ* cells not being sensitive to CPT (**Fig EV1A**). The *rad3-102 mus81Δ*
22 *yen1Δ* triple mutants were unviable (**Fig EV1C**), indicating that Yen1 also backs up Mus81 in
23 *rad3-102* cells. Finally, we found that *rad3-102 mus81Δ pol32Δ* mutants were unviable and
24 *rad3-102 mus81Δ rad51Δ* cells had a severe growth defect (**Fig 2D**). These results indicate
25 that Mus81 is required for repair mediated by both Rad51 and the polymerase δ subunit Pol32
26 in *rad3-102* cells.

28 **Mus81 and Pol32 are not required for replication progression upon Top1 poisoning**

29 Next, we reasoned that if an increased CPT sensitivity is associated with replication
30 defects, we should observe an impaired progression through S phase. Opposite to other
31 cellular models, it was reported that an acute exposure to CPT in liquid cultures of *S.*
32 *cerevisiae* cells did not induce a delay in S phase progression but rather a prolonged arrest in
33 G₂/M [50,51,73]. CPT is highly insoluble in culture media and the water-soluble derivatives
34 of CPT (topotecan and irinotecan) do not affect yeast cells growth nor their survival [74], thus

1 not being exploitable for our study. We suspected a permeability issue for CPT entry into the
2 cells, as described for the proteasome inhibitor MG132 and the DNA polymerase inhibitor
3 Aphidicolin [75,76]. We therefore used a modified culture medium described to render cells
4 more permeable to the antifungal agent brefeldin A [77] in order to increase the cell
5 permeability to CPT. We took advantage of the natural blue fluorescence emitted by the CPT
6 compound when excited by 350 nm UV light to quantify the relative amount of CPT inside
7 cells. Incubation of an asynchronous cell culture with CPT for 30 minutes in MPD +SDS
8 medium (minimal-proline-dextrose + 0.003% SDS) [77] allowed the detection of blue
9 fluorescence inside cells, whose quantification was up to 10 times higher than in cells
10 incubated with CPT for 30 minutes in standard MAD medium (minimal-ammonium-dextrose)
11 (**Fig EV2A and B**). We then used these conditions to analyze the progression through a
12 single S phase in the presence of CPT by flow cytometry. Wild type cells were synchronized
13 in G₁ with α -factor and incubated with CPT for 1 hour, then released from G₁ into S phase,
14 still in the presence of CPT. As observed by others, CPT did not alter S phase progression
15 when cells were grown in normal MAD medium (**Fig EV2C**) [50,51,73], but when incubated
16 with CPT in MPD +SDS medium, they progressed significantly slower through S phase than
17 control wild type cells incubated with DMSO, thus reaching the G₂ phase later (**Fig EV2C**).
18 Of note, the addition of SDS in the culture medium did perturb the G₁-S transition, as
19 previously reported with higher concentrations of SDS [78]. Thus, these experimental
20 conditions allowed us to perform the first characterization of the effect of CPT on DNA
21 replication using *S. cerevisiae* cells as a model system.

22 Using these conditions, we next asked how the HR factors Rad52 and Rad51, the
23 Mus81 resolvase and the polymerase δ subunit Pol32 contribute to the progression of
24 replication forks encountering poisoned Top1. At the global level, using flow cytometry, we
25 could observe that the S phase delay was strikingly increased in cells lacking one of the two
26 main HR factors, Rad52 or Rad51, compared to wild type cells (**Fig 3A**). However, in the
27 absence of Mus81 or Pol32, S phase progression was as affected by CPT as in wild type cells.
28 We confirmed these results using the *rad3-102* mutation. We synchronized cells in G₁ phase
29 with α -factor and irradiated them with UV-C. Cells were kept for 2 hours in G₁ before release
30 into S phase, in order to let compromised NER leave DNA gaps or nicks bound by the TFIID
31 complex in the *rad3-102* mutant, as described previously [65]. As expected, UV irradiation
32 only affected S phase progression in *rad3-102* cells and to a greater extent in *rad3-102* cells
33 lacking the HR factor Rad51 (**Fig 3B**). However, *rad3-102* cells lacking Mus81 or Mus81
34 nuclease activity did not show an increased S phase delay compared with *rad3-102* single

1 mutants (**Fig 3C**). All these results indicate that HR factors Rad52 and Rad51 are required to
2 promote S phase progression when replication stress is induced by CPT. However, neither
3 Mus81 nor Pol32, are required for bulk DNA synthesis in these conditions.

4 To confirm these data at the molecular level, we analyzed nascent DNA synthesis by
5 DNA combing from asynchronous cell cultures treated with CPT. DNA combing allows
6 monitoring replication fork progression genome-wide though at the level of individual DNA
7 molecules [79,80]. After incubation with CPT for 2 hours, cells were pulse-labeled with the
8 thymidine analog EdU for 20 minutes before combing analysis (**Fig 4A**). We first compared
9 the length of the EdU tracks in all strains under unchallenged conditions (+DMSO). Only
10 *mus81Δ* cells showed a significant decrease of the EdU track length compared to wild type
11 cells (**Fig 4A**; *P*-value = 0.0015). This result agrees with a recent report that proposed a role
12 for human Mus81 in DNA replication in the absence of exogenous damage [81]. Then, we
13 focused our attention onto the effect of CPT on fork progression in each strain. As expected
14 from cell cycle analyses (**Fig 3A**), CPT treatment significantly affected fork progression in all
15 strains compared to the DMSO control (**Fig 4A**). The EdU track length of wild type cells was
16 reduced by 47% and that of mutant cells was further reduced to 55% in *rad51Δ* cells and 75%
17 in *rad52Δ* cells (**Fig 4A**). However, the reduction in fork progression caused by CPT in
18 *mus81Δ* (45%) and in *pol32Δ* (37%) cells was more similar to that of wild type cells (**Fig**
19 **4A**). To accurately evaluate the effect of CPT in mutants compared to the wild type, we
20 calculated a conversion factor to equal the median of each DMSO-treated samples from
21 mutants to that of the wild type. We then used this normalization factor for each mutant to
22 convert all track length values of CPT-treated samples. The statistical analysis of the
23 normalized data show that only *rad52Δ* and *rad51Δ* mutants are more affected by CPT than
24 wild type cells (**Fig EV3A**). Our results thus argue against a role of Mus81 in replication
25 restart in CPT-treated cells, as was suggested in human cells [54]. This was confirmed in
26 *rad3-102 mus81Δ* cells, in which replication fork progression assayed by DNA combing was
27 not more affected than in single mutants (**Fig EV3B**). Overall, our results show that CPT-
28 mediated Top1 poisoning induces a global replication stress that requires HR factors for
29 replication fork progression. However, Pol32 is not required to promote fork progression in
30 response to CPT, suggesting that the strong reduction of nascent DNA tracks in *rad52Δ* and
31 *rad51Δ* mutants is not due to the inability of blocked forks to perform BIR-mediated DNA
32 synthesis. These results indicate that Pol32-dependent BIR synthesis is not the main pathway
33 promoting the progression of replication forks blocked by Top1ccs. Even though we do not

1 exclude that HR may promote the restart of blocked forks by BIR, we propose that HR is
2 primarily required to protect blocked forks from degradation in response Top1 poisoning.

4 **Mus81 resolves S phase-induced recombination events in G₂/M**

5 Our data indicate that Mus81 is required to cope with the replication stress induced by
6 Top1 poisoning but it does not promote replication progression in S phase. In human cells,
7 Mus81 has been proposed to promote fork restart by cleaving stalled or reversed replication
8 forks [54]. To understand this apparent contradiction and define the precise window of Mus81
9 activity, we assessed replication progression by two-dimensional (2D) neutral-neutral gel
10 electrophoresis in synchronized cultures after release from G₁ phase, which was previously
11 used to characterize the replication defects in *rad3-102* cells. We studied the early replication
12 origin *ARS305* and the passively replicated region C besides it [82]. Notably, *rad3-102* cells
13 accumulated complex branched structures at region C (**Fig 4B and C**, see arrows), described
14 as recombination or fork reversal events [65]. This assay gave us the opportunity to study the
15 role of Mus81 in replication fork progression and recombination intermediate resolution in
16 the *rad3-102* background. As previously described, firing at *ARS305* occurs slightly earlier in
17 *rad3-102* cells compared to wild type [65]. In the absence of Mus81 (*mus81Δ* and *rad3-102*
18 *mus81Δ* mutants), slower replication was observed around the *ARS305*, as the Y arc signal
19 was still clearly observable at 60 minutes, while it had already disappeared in wild type and
20 *rad3-102* cells (**Fig 4C**, left panel). This is consistent with a slower replication fork
21 progression in *mus81Δ* than in wild type cells observed in the absence of CPT-induced DNA
22 damage (**Fig 4A and Fig EV3B**). Complex branched structures did not accumulate in region
23 C in the absence of Mus81 in *rad3-102 mus81Δ* cells (**Figure 4C**, see arrows and **Fig**
24 **EV3D**), suggesting that Mus81 does not process these substrates, observed in *rad3-102* cells
25 during S phase. More interestingly, we noted that, in a 100 minutes time-window after G₁
26 release, *ARS305* fired twice in wild type, *mus81Δ* and *rad3-102* cells, implying two rounds of
27 replication. This was not observed in the *rad3-102 mus81Δ* mutant (**Fig 4C**, left panel). As
28 for *ARS305* region, replication forks progressed only once through region C in *rad3-102*
29 *mus81Δ* cells (**Figure 4C**, right panel). These results made us consider a cell cycle delay that
30 could stem from a replication termination defect in the absence of Mus81 in *rad3-102* cells.

31 To explore this, we monitored the appearance of YFP-Rad52 foci in cells exposed to
32 CPT (**Fig 5A**). Wild type cells were synchronized in G₁ with α -factor and incubated with CPT
33 for 30 minutes, then released from G₁ into S phase in the presence of CPT. Wild type cells
34 incubated with DMSO showed the appearance of a low amount of Rad52 foci in S phase 40

1 and 60 minutes after release from G₁ (Fig 5C). These foci disappeared when cells reached
2 G₂/M (Fig 5B and C), consistent with the observation that spontaneous Rad52 foci
3 predominantly form in S phase cells [83]. When wild type cells were released from the G₁
4 arrest in the presence of CPT, they accumulated 6 times more Rad52 foci than in control cells
5 only when cells entered S phase at 40 minutes (Fig 5B and C). This accumulation
6 continuously increased until cells reached the G₂/M phase at 80 minutes and then started to
7 decrease in later time points (Fig 5B and C). These results show that S phase entry is
8 required for the initiation of recombination events induced by CPT and these events are
9 resolved after the completion of DNA replication in G₂/M. When assessing the contribution
10 of Mus81, we made two main observations. First, the increased accumulation of Rad52 foci
11 during S phase caused by CPT exposure was not suppressed in the absence of Mus81 (Fig 5B
12 and C), suggesting that Mus81 is not required to generate the substrates for recombination.
13 Second, we observed that CPT-induced Rad52 foci in *mus81Δ* cells accumulated over the
14 entire time course experiment until 150 minutes, not showing the decrease observed in wild
15 type cells during the G₂/M phase (Fig 5B and C). This phenotype was again confirmed in
16 *rad3-102 mus81Δ* cells, which accumulated more Rad52 foci than either single mutant (Fig
17 EV4A). These results suggest that Mus81 processes S phase-induced recombination events in
18 G₂/M.

19 In an unperturbed cell cycle, the regulating subunit Mms4 of the Mus81-Mms4
20 complex is hyper-phosphorylated by multiple kinases at the G₂/M transition, and this
21 correlates with an enhanced activity of the complex on branched DNA structures *in vitro* [58-
22 61]. To validate if the temporal regulation of Mus81 activity fits with the timely requirement
23 for Mus81 to process recombination intermediates generated during CPT-induced damage
24 repair (Fig 5C), we followed a synchronous culture going through a single S phase and
25 analyzed Mms4 phosphorylation. Treatment of wild type cells with CPT caused a delay in S
26 phase progression with respect to control cells, with a consequent delay in Mms4
27 phosphorylation, detected as an electrophoretic mobility shift by immunoblotting (Fig 5D).
28 The highest degree of Mms4 phosphorylation was reached 90 and 120 minutes after G₁
29 release in control cells and cells treated with CPT, respectively (Fig 5D). In *rad52Δ* cells
30 exposed to CPT, cells arrested in late S phase and no phosphorylation of Mms4 was observed
31 (Fig EV4B). We also analyzed Mms4 phosphorylation in wild type and *rad3-102* cells. First,
32 it is worth noting that exposure to UV-C in G₁ phase did not induce any change of Mms4
33 mobility in both tested strains (Fig EV4C). In wild type cells, Mms4 underwent
34 phosphorylation 60 to 70 minutes after release from G₁, when most cells had reached the

1 G₂/M phase. However, no phosphorylation of Mms4 was observed in *rad3-102* cells, in
2 agreement with their accumulation in S phase after G₁ release (**Fig EV4C**). Thus, the nuclease
3 activity of Mus81-Mms4 is required to process S phase-associated HR events once the cells
4 reach G₂/M (validated by the accumulation of the mitotic cyclin B2 (Clb2)) (**Fig 5D**). Indeed,
5 hyper-phosphorylation of Mms4 is required for the function of Mus81 in the repair of CPT-
6 induced DNA damage, as indicated by the increased sensitivity to CPT compared to wild type
7 cells of the *mms4-14A* mutant, which cannot undergo phosphorylation by Cdk1 and Cdc5, and
8 of the *cdc5-2* kinase-defective mutant (**Fig 5E and Fig EV4D**) [59]. However, we noticed
9 that the CPT sensitivity of the *mms4-14A* mutant was lower than in the complete absence of
10 Mms4 (*mms4Δ*), and the *mms4-9A* mutant (*mms4- η* , [58]) was not found more CPT sensitive
11 than the wild type (**Fig EV4D**). This discrepancy between *mms4* mutants could be explained
12 by the high number and redundancy of Mms4 phosphorylation sites [59,61].

13 Last, to confirm the need of Mus81 only after the completion of genome duplication,
14 we limited the availability of Mus81 to the S or G₂/M phases by taking advantage of the
15 regulatory elements of the cyclin B6 and B2, respectively [84,85]. Fused to the S-tag [85],
16 Mus81 was only present when cells entered the S phase and was targeted for degradation
17 when reaching the G₂/M phase (**Appendix Fig S5A**). Fused to the G₂/M tag [84], Mus81
18 appeared once cells reached the G₂/M phase and was targeted for degradation in the following
19 G₁ phase (**Appendix Fig S5B**). Cells bearing the *S-MUS81* allele were sensitive to CPT-
20 induced DNA damage, similar to cells lacking Mus81, Mms4 or the catalytic activity of
21 Mus81 (*mus81-dd*) (**Fig 5E**). On the contrary, cells bearing the *G₂/M-MUS81* allele were not
22 sensitive to CPT-induced DNA damage (**Fig 5E**). Analogously, *rad3-102 S-MUS81* cells
23 were more sensitive to UV than the *rad3-102* single mutant, whereas *rad3-102 G₂/M-MUS81*
24 were not (**Fig 5F**).

25 Overall, we conclude that HR factors are required for the restart of replication forks
26 blocked by Top1 poisoning by mediating recombination events. Despite that these events
27 occur in S phase, Mus81 nuclease is acting to process recombination events only when its
28 activity is increased during the G₂/M phase. Our results also show that Mus81 was not
29 required for the assembly of Rad52 foci, nor was activated at the time of their appearance,
30 indicating that Mus81 is unlikely to be involved in the generation of HR substrates by
31 cleaving replication forks in our systems.

32

33 **Mus81 processes recombination intermediates to complete replication**

1 Our 2D-gel analysis indicated that *rad3-102 mus81Δ* cells synchronously released
2 from a G₁ block had a delay in starting the following cell cycle (**Fig 4C**). We confirmed this
3 observation in cells exposed to Top1 poisoning by performing longer time course flow
4 cytometry experiments. Indeed, *mus81Δ* cells treated with CPT started the following cell
5 cycle 40 minutes later than wild type cells under the same treatment (**Fig EV5A**). This cell
6 cycle delay could stem from the inability of cells to timely process recombination
7 intermediates induced by replication fork restart. Since we proposed that HR may promote the
8 restart of CPT-induced blocked forks by BIR, this implies the formation of a D-loop. Merging
9 of the D-loop with a converging replication fork would form a single Holliday junction,
10 whose resolution would be mandatory for replication termination. We did not observe an
11 accumulation of recombination or termination intermediates in *rad3-102 mus81Δ* cells, yet
12 they may have not accumulated in the region analysed by 2D-gels. Thus, we decided to look
13 for the accumulation of termination intermediates at the genome level by pulsed-field gel
14 electrophoresis (PFGE). PFGE allows the separation of individual chromosomes according to
15 their size in G₁ and G₂/M cells. However, when cells enter S phase, the chromosomes are
16 trapped into the wells due to the presence of joint molecules (JMs), as replication bubbles and
17 other replication intermediates. For instance, this could be observed for wild type or *mus81Δ*
18 cells incubated with DMSO, for which chromosome bands disappeared from the gel 40
19 minutes after the release from G₁ and could be detected back again at 60 minutes (**Fig 6B**,
20 upper panel). After Southern blot analysis, we could observe that chromosome IV was
21 retained in the gel well at S-phase entry 40 minutes after release and then progressively
22 disappeared from the wells (**Fig 6B**, lower panel). This is consistent with the FACS analysis,
23 which shows that bulk DNA synthesis started at 40 minutes and ended 60 to 80 minutes after
24 release (**Fig 6A**). Incubation of wild type cells with CPT induced a delay in S phase
25 progression (**Fig 6A**), which was mirrored by the kinetics of accumulation of JMs in the gel
26 wells (**Fig 6B**). As shown before (**Fig 3A**), CPT treatment induced the same S phase
27 progression delay in *mus81Δ* and wild type cells (**Fig 6A**). Yet, opposite to the situation in
28 wild type cells, the amount of JMs did not decrease 100 minutes after release from G₁ in
29 CPT-treated *mus81Δ* cells and chromosomes hardly re-entered the gel (**Fig 6B**). Since the
30 bulk of DNA synthesis had already ended at that time, these JMs unlikely represents
31 replication forks but rather single Holliday junctions that would form upon the merging of D-
32 loops with converging forks. The *cdc5-2* mutant fails to phosphorylate the Mus81-Mms4
33 complex in G₂/M in order to increase its nuclease activity [59]. In CPT-treated *cdc5-2* cells,
34 we observed a similar accumulation of JMs after the bulk of DNA replication compared to the

1 *mus81Δ* mutant, suggesting that Mus81-Mms4 complex phosphorylation in G₂/M is required
2 for the nuclease to resolve CPT-induced JMs (Fig 6B). In G₂/M-MUS81 cells, in which
3 Mus81 presence is strictly restricted to the G₂/M phase (Appendix Fig S5B), CPT-induced
4 JMs did not accumulate over time, indicating that the restriction of Mus81 to G₂/M is
5 sufficient for JMs processing (Fig 6B). Altogether, these results indicate that Mus81 nuclease
6 activity would be required in G₂/M for the processing of termination intermediates that
7 specifically form after the restart / protection of replication forks by HR during S phase.

8 One last remaining question was whether this BIR-mediated replication restart occurs
9 from broken or reversed replication forks. The stabilization of a nicked DNA bound to Top1
10 by CPT has been initially proposed to induce a replication fork run-off, leading to the
11 formation of a one-ended DSB [49]. More recently, an alternative model proposed that Top1
12 inhibition by CPT causes the accumulation of topological constrains in DNA that may impede
13 the progression of replication forks [50,53]. Since 20 to 25% of forks have been observed to
14 be reversed by electron microscopy after *in vivo* psoralen crosslinking in CPT-treated yeast
15 cells, it was suggested that the accumulation of supercoils in front of replication forks
16 promote their reversal [50,52]. Reversed forks are formed upon the annealing of nascent
17 strands together, exposing a single double-strand DNA end that mimics a one-ended DSB. In
18 PFGE experiments, the occurrence of DSBs would result in the diminution of full-length
19 chromosome bands and the appearance of smeared signals. This could be readily observed
20 wild type cells released in S phase in the presence of the radiomimetic agent zeocin, which
21 induces fragmentation of chromosomes (Fig EV5B). We could not observe such smears in
22 wild type CPT-treated cells when they progressed through S phase 30 to 60 minutes after
23 release from the G₁ block (Fig 6B and Fig EV5B). Similarly, we could not observe any
24 smeared signal in S-phase *rad3-102* cells (Fig EV5C). In the absence of Rad52, which is
25 strictly required for HR-mediated repair of DSBs, smears could be detected only when CPT-
26 treated cells reached the G₂ phase 100 minutes after release (Fig 6C and D). This could be
27 explained by the accumulation of unrepaired CPT-induced DSBs that could not be detected in
28 S phase. Alternatively, the appearance of broken chromosomes in G₂ *rad52Δ* cells could be
29 the consequence of the cleavage of blocked or reversed forks that could not undergo restart
30 through HR. The latter hypothesis is supported by densitometry analysis of PFGE, showing
31 that the amount of smeared signals at 140 minutes post-release in *rad52Δ* cells treated with
32 CPT was reduced by 50% in the absence of the Mus81 nuclease (Figure 6C and D).

33 Altogether, our results favour a model in which Top1 poisoning by CPT in our
34 experimental conditions mostly induces replication fork reversal rather than DSBs during S

1 phase. Mus81 is required to process recombination intermediates that accumulate at
2 termination sites following HR-mediated replication forks restart and forks that could not be
3 restarted because of an HR defect.

8 **DISCUSSION**

10 In the present study, we have used Top1 poisoning by CPT to generate a replication
11 stress genome-wide and to study the restart of replication forks blocked by Top1ccs. Our
12 results do not support a model involving Pol32-dependent BIR to promote replication
13 progression at the global level. However, HR factors Rad52 and Rad51 are required during S
14 phase to protect blocked forks from degradation. We propose that HR mainly acts by
15 generating a D-loop structure protecting blocked forks until their merging with convergent
16 forks. The Mus81 nuclease does not participate in the replication restart but appears to be
17 essential to resolve recombination intermediates to promote the termination of
18 protected/restarted forks. Thanks to a complementary, though independent, approach using
19 the *rad3-102* allele, we conclude that this mechanism is independent of the accumulation of
20 DNA supercoiling and DNA-protein crosslinks naturally caused by CPT.

21 It is worth emphasizing that this work has been made possible because we set up
22 conditions to improve CPT entry into yeast cells in liquid cultures. Using standard culture
23 conditions, CPT treatment did not detectably delay S phase completion, even in *rad52Δ*
24 mutants, whereas this phenotype was clearly observed in CPT-treated human cells [50]. The
25 culture conditions we used allowed observing a CPT-dependent S phase delay, which could
26 be exacerbated by mutants defective in HR as *rad52Δ* and *rad51Δ*. These S phase progression
27 defects could be characterized at the molecular level by demonstrating replication defects by
28 both DNA combing and pulsed-field gel electrophoresis (PFGE) experiments. The latter
29 experiments could also show physical evidence of chromosome breakage upon CPT
30 treatment, which was not found in previous studies [50,51]. Nevertheless, DSBs only
31 appeared in CPT-treated *rad52Δ* cells and not during their progression through S phase, as
32 expected from the conversions of nicked DNA into DSBs by the passage of replication forks
33 [47-49]. DSBs appeared when cells reached the G₂/M phase and were partially dependent on
34 the presence of the Mus81 nuclease. The remaining DSBs are expected to be dependent on the

1 Yen1 nuclease because the survival of *mus81Δ* cells is sustained by the presence of Yen1 in
2 response to CPT and in *rad3-102* cells [71,72]. Consistently, Yen1 catalytic activity is
3 restricted to mitosis [59,86,87].

4 CPT-induced DSBs were also detected in human cells and were partially Mus81-
5 dependent [54]. In these cells, immunostaining of γ -H2AX, a marker of DNA damage
6 checkpoint activation, was co-localizing with sites of neo-synthesized DNA. This led the
7 authors to propose that Mus81 cleaves stalled replication forks to promote their restart [54].
8 However, these experiments were performed in HCT116 colorectal carcinoma cells, which
9 are deficient for Mre11 [88,89]. *mre11Δ* yeast cells are as sensitive to CPT as *rad52Δ* cells
10 and the combinations of *mre11Δ* or *rad52Δ* with *rad3-102* are lethal [65], suggesting that
11 Mre11 is absolutely required for HR-mediated replication restart. Thus, the results described
12 in human HCT116 cells are reminiscent of the Mus81-dependent DSBs we have observed in
13 *rad52Δ* cells, which likely represent the cleavage of blocked or reversed forks that could not
14 undergo restart through HR (**Fig 7**) [90].

15 Our results are consistent with a recent study on the survival to an inducible,
16 replication-born, one-ended DSB, which primarily depends on Rad52 and Rad51 [4]. Since
17 we did not detect DSBs in wild-type CPT-treated nor in *rad3-102* cells, we favor the
18 hypothesis that replication restart in our systems would use the tip of reversed forks as
19 substrates for HR, as proposed in fission yeast and in mammalian cells [32,40]. This would
20 explain why the absence of the Ku complex, which is normally recruited to double-stranded
21 DNA ends, could suppress the resection defect caused by the lack of Mre11 nuclease activity in
22 cells treated with CPT or containing the *rad3-102* allele [66]. Additionally, replication
23 intermediates corresponding to fork reversal were observed by 2D-gel analyses in *rad3-102*
24 cells [65]. The occurrence of fork reversal in *rad3-102* cells would suggest that fork reversal
25 upon Top1 poisoning would not be the consequence of the accumulation of topological stress
26 ahead of replication forks [50,53,91]. DNA end resection of reversed forks would involve the
27 Mre11 and Exo1 nucleases [7,92,93] and Rad52 and Rad51 would promote the efficient
28 invasion of the parental duplex (**Fig 7**).

29 We had previously reported that replication restart in the absence of Rad51 in *rad3-*
30 *102* cells can still be compensated by the presence of Pol32, a non-essential subunit of DNA
31 polymerase δ [65]. The repair of replication-born DSBs by sister-chromatid recombination in
32 a plasmid-based system confirmed the existence of a Rad51-independent, Pol32-dependent
33 pathway [68]. A similar pathway, termed MIDAS, has been described in mammalian cells
34 and depends on RAD52 and POLD3, the homolog of Pol32, but not on RAD51 [94-96]. Here,

1 we have shown that Pol32 also compensates the absence of Rad51 for CPT resistance and
2 DSB repair by BIR. These results have led us to propose that Pol32 could stimulate strand
3 invasion in the absence of Rad51. The absence of Pol32 alone only affects cell survival in
4 response to high doses of CPT [69]. Likewise, Pol32 is not primarily required for cell survival
5 in response to broken replication forks in the Flp-nick system [4]. The Pif1 DNA helicase is
6 required for extensive DNA synthesis during BIR [24,26]. However, the absence of nuclear
7 Pif1 in *pif1-m2* mutants does not sensitize cells to CPT [4], nor leads to lethality in *rad3-102*
8 *rad51Δ* cells (unpublished observations). These results suggest that extensive BIR-mediated
9 synthesis is not essential in response to blocked forks. Because DNA replication is initiated
10 from multiple origins along chromosomes, blocked replication forks could be restarted by
11 BIR but rapidly stopped by a convergent fork (Fig 7) [4]. Nevertheless, HR events are
12 occurring in S phase in response to CPT and HR factors required for strand invasion, Rad52
13 and Rad51, are essential to complete replication in response to blocked forks. Track length of
14 nascent DNA synthesis are reduced in the absence of these factors, suggesting that HR
15 engagement could protect nascent strands from degradation until encountering a convergent
16 fork (Fig 7). Consistently, it has been shown that nascent DNA strands at blocked or broken
17 forks are extensively degraded by nucleolytic activities in the absence of Rad52 in fission
18 yeast or BRCA2 in human cells [7,8,18,90], preventing the merging with a convergent fork
19 [7]. This protection mechanism could happen through the formation of a D-loop, a
20 mechanism reminiscent of the protection of chromosome ends by the looping of telomeres (T-
21 loops) [97].

22 Mus81 is required for cell survival in response to CPT and in the *rad3-102*
23 background. Our molecular analyses show that Mus81 is not required for HR-mediated restart
24 of blocked forks but rather for the processing of joint molecules after the bulk of DNA
25 synthesis in G₂/M phase. After the restart of a broken fork by BIR, it has been proposed that
26 Mus81 could cleave the migrating D-loop in order to limit the mutagenesis associated with
27 BIR synthesis and re-establish a stable fork structure [4]. Our data argue against the
28 possibility that Mus81 could fulfil this role during the S phase because Mus81 is not activated
29 in S phase by the replication stress caused by CPT or *rad3-102* and the absence of Mus81
30 does neither affect, nor ameliorate, replication progression in these conditions. One possibility
31 is that HR-mediated replication restart is initiated in S phase but the priming of DNA
32 synthesis is delayed until G₂/M, when Mus81 catalytic activity is enhanced by the hyper-
33 phosphorylation of its regulatory partner Mms4. This hypothesis is consistent with the
34 observed time delay between strand invasion and the initiation of DNA synthesis within the

1 D-loop when BIR initiated in G₂/M [21]. In the context of fork restart, this delay would give
2 the opportunity to a convergent fork to reach the blocked forks **protected** by HR to ensure the
3 completion of replication without challenging genome stability. The fusion of a D-loop with a
4 convergent replication fork would lead to the formation of a nicked Holliday junction, for
5 which the Mus81 **nuclease** has a high affinity *in vitro* [98,99]. **Indeed, we observed in cells**
6 **treated with CPT, and devoid of Mus81 activity, an accumulation of joint DNA molecules,**
7 **which are consistent with the formation of Holliday junctions. Thus, in response to fork**
8 **blockage, Mus81 could process Holliday junctions at termination sites (Fig 7), as suggested**
9 **previously [4]. We propose that this would be the essential function of Mus81 after the restart**
10 **of blocked forks.** During the preparation of this manuscript, and by using cell cycle-regulated
11 Mus81 constructs as we did in this work, the Pfander lab has come to the same conclusion
12 that Mus81 acts in a post-replicative manner in response to various replication stresses
13 (Bittmann et al., 2020).

14

15 In conclusion, our work describes how cells deal with blocked replication forks
16 dispersed throughout the genome by Top1 poisoning. Although we do not exclude that HR
17 factors Rad52 and Rad51 can orchestrate the restart of DNA synthesis by BIR, these factors
18 primarily act to protect blocked forks until they merge with converging forks. The processing
19 of intermediates formed during fork merging by Mus81 is then required for the completion of
20 DNA replication. Because incomplete NER reactions as those observed in *rad3-102* cells also
21 occur in wild type cells [100], and Top1ccs have been found to accumulate naturally [44], this
22 mechanism is likely to be general in response to other replication blocks requiring HR to
23 complete replication. Finally, the method we have described to use CPT in yeast cell cultures
24 will allow performing a further characterization of Top1 poisoning by CPT at the molecular
25 level. This will indubitably have important implications for understanding the effects of CPT
26 as a chemotherapeutic agent.

1 MATERIALS AND METHODS

2

3 Yeast strains

4 All *Saccharomyces cerevisiae* yeast strains used in this study are in W303-1aR5 background
5 (*his3-11, 15 leu2-3, 112, trp1-1 ura3-1 ade2-1 can1-100 RAD5*) unless indicated and are
6 listed in table EV1. Deletion mutants were either obtained by the PCR-based gene
7 replacement method (verified by PCR and drug sensitivity assays) or by genetic crosses
8 (verified by tetrad analysis).

9

10 Sensitivity assays to camptothecin (CPT) and ultra-violet irradiation (UV)

11 Cells from mid-log cultures were counted using a CASY[®] (OLS system) and concentrated to
12 1×10^8 cells/mL, 10 μ L of 10-fold serial dilutions were spotted on rich YPAD plates +/- CPT
13 ((S)-(+)-Camptothecin, Sigma #C9911) and plates were incubated for 3 days at 30°C. To
14 assay UV sensitivity, 10-fold serial dilutions were spotted on YPAD plates and plates were
15 irradiated with UV-C in a Bio-Link[™] BLX crosslinker and incubated for 3 days at 30°C in
16 darkness. To quantify cell growth from drop tests, the box tool from Image J software was
17 over-imposed onto the five/six drops corresponding to each strain and the area under the
18 curve of the derived plots was retrieved. Raw area values were directly exploited in GraphPad
19 Prism for quantification and statistical analyses. Three to four independent biological
20 replicates have been performed for each sensitivity assay.

21

22 Colony-forming unit assays

23 Cells from mid-log cultures were counted using a CASY[®] (OLS system), concentrated to
24 1×10^7 cells/mL, and appropriate dilutions were plated on rich YPAD plates +/- CPT ((S)-(+)-
25 Camptothecin, Sigma #C9911). Survival rates were determined by counting colonies after
26 incubating the plates for 3 to 5 days at 30°C. Four independent biological replicates have been
27 performed for each genotype assayed.

28

29 Cell cycle progression analyses

30 Overnight mid-log cultures at 7×10^6 cells/mL were synchronized in G₁ with α -factor (0.5
31 μ g/mL) in YPD medium for 2 to 3 hours at 30°C. For UV-induced DNA damage, G₁-
32 synchronized cells were resuspended in water onto Petri dishes as a 4mm-deep cell
33 suspension, irradiated with UV-C in a Bio-Link[™] BLX crosslinker, resuspended again in
34 YPD medium with α -factor, incubated for 2 more hours in darkness and released into S phase

1 by addition of pronase (50 µg/mL). For CPT-induced DNA damage, G₁-synchronized cells
2 were washed with MPD+SDS medium (0.17% yeast nitrogen base, 0.1% L-proline, 2%
3 glucose and 0.003% SDS) [77], resuspended in MPD+SDS medium supplemented with α-
4 factor and DMSO or CPT, incubated for 1 more hour and released into S phase by addition of
5 pronase (50 µg/mL). Samples (430µL) were taken every 10 or 15 minutes and fixed with
6 100% ethanol for subsequent flow cytometry analysis. Cells were centrifuged for 1 minute at
7 16.000 RCF, resuspended in 50 mM sodium citrate buffer containing 10 µl of RNase A (20
8 mg/ml, Qiagen 76254) and incubated for 2 hours at 50°C. Then, 10 µl of proteinase K
9 (Sigma, P6556) were added for further incubation 2 hours at 50°C. Aggregates of cells were
10 dissociated by sonication. 20 µl of cell suspension were incubated with 200 µl of 50 mM
11 sodium citrate buffer containing 4 µg/mL propidium iodide (Sigma) for at least 2 hours in
12 darkness. Data were acquired on a MACSQuant analyzer (Miltenyi Biotech) and analyzed
13 with FlowJo software. Two to four independent biological replicates have been performed for
14 each cell cycle progression analysis.

15

16 **DNA combing**

17 DNA combing was performed essentially as described [79]. EdU incorporation and uptake in
18 cells was realized in strains bearing 7 integrated copies of the *Herpes simplex* virus thymidine
19 kinase (HSV-TK) and the human equilibrative nucleoside transporter 1 (hENT1) on a
20 plasmid. Overnight mid-log cultures at 5x10⁶ cells/mL in YPD medium were washed with
21 MPD +SDS medium and resuspended in MPD +SDS with DMSO or 50µM CPT, incubated
22 for 2 hours and pulse-labeled with 50µM EdU for 20 minutes. DNA fibers were combed on
23 silanized coverslips. Total DNA was detected with YOYO[®]-1 iodide (Molecular Probes
24 Y3601) and EdU-labeled DNA was detected by Click chemistry using 20µL of the following
25 mix per coverslip: 16.7 µL of H₂O, 0.8 µL of 100 mM Copper sulfate, 0.5 µL of 0.5 mg/mL
26 Alexa Fluor[®] 555 Azide (Molecular Probes A20012) and 2 µL of 100 mM Sodium ascorbate.
27 Coverslips were incubated for 1 hour at 60°C in a humid chamber in obscurity. Images were
28 recorded on a Leica DM6000 microscope equipped with a CoolSNAP HQ CCD camera
29 (Roper Scientific) and processed as described [101]. For comparing track length values of
30 CPT-treated samples, the median values of all DMSO-treated mutant samples were
31 normalized to the median value of the wild-type strain as to obtain a normalization factor. For
32 each mutant, we have used this normalization factor to convert all track length values of CPT-
33 treated samples. For statistical analysis, we used a Mann-Whitney test to compare, in each

1 case, two unpaired groups with no Gaussian distribution. Two independent biological
2 replicates were performed, [summing at least 100 counted EdU tracks per condition](#).

3

4 **Protein analyses**

5 For time course experiments, proteins were extracted from cell pellets with acid-washed glass
6 beads in a denaturing cracking buffer (8M Urea, 5% SDS, 40 mM Tris-HCl pH 6.8, 0.1 mM
7 EDTA, 0.4 mg/mL bromophenol blue, 50 mM NaF, 150 mM β -mercaptoethanol, 2 mM
8 PMSF) supplemented with cOmplete™ (Roche) protease inhibitors, 20 minutes at 70°C under
9 permanent agitation at 1400 rpm in a Thermomixer® (Eppendorf). Extracts were cleared by
10 centrifugation, separated in Nupage® 3-8% (Invitrogen) polyacrylamide gels and blotted on
11 PVDF membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Two to three
12 independent biological replicates have been performed for each time course experiment.

13 For immunodetection, the following antibodies were used: anti-FLAG® M2 (Sigma-Aldrich,
14 F1804), anti-Clb2 (Santa Cruz Biotech, y-180), [anti-HA \(Abcam, ab9110\)](#) and anti-tubulin
15 YOL1/34 (Abcam, ab6161).

16

17 **Pulsed-field gel electrophoresis**

18 Agarose plugs containing chromosomal DNA were made as described [80]. Chromosomes
19 were separated at 13°C in a 0.9% agarose gel in TBE 0.5x using a Rotaphor apparatus
20 (Biometra) using the following parameters: interval from 100 to 10 seconds (logarithmic),
21 angle from 120 to 110° (linear), voltage 200 to 150 V (logarithmic). The gel was subsequently
22 stained with ethidium bromide, and transferred to Hybond XL (GE Healthcare).
23 Quantification of chromosome intensity was performed with Imaje J software after Southern
24 blotting and hybridization using a radioactive probe specific for chromosome IV (*ARS453*) or
25 VIII (*RRM3* gene), using a PhosphorImager (Typhoon Trio, GE). Two independent biological
26 replicates were performed.

27

28 **Microscopy analyses**

29 For the visualization of CPT inside cells, images were recorded on a Leica DM6000
30 microscope after excitation of CPT compound with a 350 nm UV light. Fluorescence
31 quantification was performed with Image J software [for three independent biological](#)
32 [replicates](#). For the analysis of spontaneous Rad52 foci, non-fixed nuclei from exponentially
33 growing cell cultures bearing plasmid pWJ1344 were stained with DAPI and Rad52-YFP foci
34 were counted in S-G₂ cells. For the kinetic analyses of CPT-induced Rad52 foci, non-fixed

1 nuclei were visualized thanks to mCHERRY-Pus1 and Rad52-YFP foci were counted in all
2 cells. Four independent biological replicates were performed.

3

4 **2D gel electrophoresis**

5 For DNA extraction, 50 mL of the desired cultures were collected in Falcon tubes containing
6 500µL 10% Sodium Azide and kept in ice till processing. Cells were washed with 5 mL of
7 chilled water and carefully resuspended in 1 mL of 1M sorbitol-10mM EDTA pH 8-0,1% β-
8 mercaptoethanol-2 mg/mL Zymoliase 20T, and then incubated at 30°C during one hour under
9 soft agitation. After centrifugation, the sferoplast pellet was washed with 500 µL of cold
10 water and then broken with 400 µL of cold water plus 500 µL of 1.4M NaCl-100mM Tris-Cl
11 pH 7.6-25mM EDTA pH 8-2% CTAB and incubated for thirty minutes at 50°C with 40 µL 10
12 mg/mL RNase. Next, 40 µL 20 mg/mL Proteinase K were added and incubation performed
13 overnight under very soft agitation. After centrifugation, pellet and supernatant were treated
14 separately. The supernatant was extracted with 500 µL (24:1) Chloroform:Isoamyl Alcohol
15 and DNA precipitated with two volumes of 50 mM Tris-Cl pH 7.6-0 mM EDTA pH 8-1%
16 CTAB and further resuspended in 250 µL 1.4M NaCl-1 mM EDTA pH 8-10 mM Tris-Cl pH
17 7.6. The original pellet was resuspended in 400 µL 1.4M NaCl-1 mM EDTA pH 8-10 mM
18 Tris-Cl pH 7.6 and incubated during one hour at 50°C, extracted with 200 µL (24:1)
19 Chloroform:Isoamyl Alcohol and rejoined with the supernatant DNA. The whole sample was
20 precipitated then with one volume room temperature isopropanol, centrifuged, washed with
21 70% ethanol and resuspended in 100 µL 10mM Tris-Cl pH 8. DNA was digested using 200 U
22 of each *EcoRV* and *HindIII* during 5 hours and NaCl-precipitated. First dimension
23 electrophoresis was carried out at room temperature in 0.4% agarose gels at 40V during
24 twenty hours in 1x TBE, next stained with ethidium bromide and a band comprised between 2
25 and 12 kb was cut and rotated 90° for the second dimension electrophoresis. Second
26 dimension electrophoresis was carried out at 4°C in 1% agarose gels containing 0.34 µg/mL
27 ethidium bromide at 130V during 12 hours in 1x TBE containing 0.34 µg/mL ethidium
28 bromide. Gels were treated and transferred by standard procedures. For hybridization,
29 coordinates of α³²⁻P PCR probes were 37883-41883 for *ARS305* and 57903-61158 for region
30 C on chromosome III. Signals were acquired using a Fujifilm FLA-5100 PhosphorImager.
31 Two independent biological replicates were performed.

32

33 **BIR assay**

1 Exponentially growing cells in YPR (2% Raffinose) medium were plated on rich medium
2 containing 2% glucose (YPD) or 2% galactose (YPG) and incubated 3 days at 30°C. Colonies
3 on YPG plates were replica-plated onto synthetic complete (SC) medium lacking lysine. BIR
4 frequencies were determined by dividing the number of Lys+ by the number of YPD cfu. For
5 statistical analysis, we used a [Mann-Whitney test \(two unpaired groups, non-parametric\)](#).
6 Three to six independent biological replicates were performed for each [genotype assayed](#).

7 8 **Statistical Analysis**

9 [Statistical analyses for different experiments were done in GraphPad Prism 8. As indicated in](#)
10 [the relevant figure legends, a Mann-Whitney non-parametric test comparing unpaired groups](#)
11 [of values was applied, for no Gaussian distributions were assumed. A *P*-value of < 0.02 was](#)
12 [deemed significant. Mean ± SEM are displayed for the concerned experiments, and sample](#)
13 [size was not predetermined using any statistical method.](#)

14 15 **Data Availability**

16 [Raw data that support the findings of this study others than the source data associated to this](#)
17 [article \(e.g. microscopy images\) have not been deposited in a public database and are](#)
18 [available from the corresponding authors upon reasonable request.](#)

19
20 [Expanded view for this article is available online.](#)

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9 **AUTHOR CONTRIBUTIONS**

10 Conceived and designed the experiments: BP MMC AA PP. Performed the experiments: BP
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12

13 **CONFLICT OF INTEREST**

14 The authors declare that they have no conflict of interest.

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49

1 **FIGURE LEGENDS**

2

3 **Figure 1.** HR-mediated replication restart occurs by a BIR-like mechanism.

4 (A) (B) CPT sensitivity assayed by 10-fold serial dilutions of different mutant combinations
5 on YPAD plates. Four biological replicates have been performed.

6 (C) Colony-forming unit assays to increasing CPT concentrations of the same mutant
7 combinations as in (B). Mean values \pm SD are plotted. Four biological replicates have been
8 performed.

9 (D) UV sensitivity assayed by 10-fold serial dilutions of different mutant combinations with
10 *rad3-102* allele on YPAD plates after exposure to the indicated UV-C doses. Five biological
11 replicates have been performed.

12 (E) Schematic representation of the BIR assay. Two fragments of the *LYS2* gene sharing 2.1
13 kb of homology (*lys* and *ys2*) were integrated on chromosomes V and XI, respectively.
14 Induction of *HO* endonuclease expression under control of the *GAL1* promoter produces a
15 DSB next to the *lys* fragment that can be repaired by BIR. BIR events can be scored by
16 selecting survivor colonies, which harbor a functional *LYS2* gene.

17 (F) BIR frequencies (Lys⁺ survivors among total cells) for the WT and indicated mutant
18 strains are plotted on a logarithmic scale. Data represent the mean \pm SD from 3 to 6
19 independent experiments. ns, non-significant difference, * *P*-value = 0.0134, Mann-Whitney
20 unpaired t test.

21

22 **Figure 2.** *Mus81* is required in the restart pathway involving both *Rad51* and *Pol32*.

23 (A) (B) CPT sensitivity assayed by 10-fold serial dilutions of different mutant combinations
24 on YPAD plates. Four biological replicates have been performed.

25 (C) UV sensitivity assayed by 10-fold serial dilutions of different mutant combinations with
26 *rad3-102* allele on YPAD plates after exposure to the indicated UV-C doses. Four biological
27 replicates have been performed.

28 (D) Synthetic combinations of *rad3-102 mus81* Δ with *rad51* Δ and *pol32* Δ . Tetrads dissected
29 on YPAD medium are shown. Triangles indicate either a severe growth defect or lethality.

30

31 **Figure 3.** *Mus81* is not required for S phase progression in CPT-treated and *rad3-102* cells.

32 (A) Analysis of DNA content by flow cytometry of G₁ phase synchronized wild type, *rad52* Δ ,
33 *rad51* Δ , *mus81* Δ and *pol32* Δ cells and further released into S phase. Cells were synchronized

1 in G₁ with α -factor, treated with DMSO or 100 μ M CPT, let in G₁ for 1 h, and released into S
2 phase. Three biological replicates have been performed.

3 **(B) (C)** Analysis of DNA content by flow cytometry of G₁ phase synchronized wild type,
4 *rad3-102*, *rad3-102 rad51 Δ* , *rad3-102 mus81* and *rad3-102 mus81-dd* cells and further
5 released into S phase. Cells were synchronized in G₁ with α -factor, untreated or irradiated
6 with 20 J/m² UV-C, let in G₁ for 2h, and released into S phase. Three biological replicates
7 have been performed.

8 **Data information:** Asterisks indicate the progression of cells in S phase.

9

10 **Figure 4.** Rad52 and Rad51 are required for HR-mediated replication fork protection.

11 **(A)** Analysis of replicated DNA tracks length by single-molecule DNA combing in WT,
12 *rad52 Δ* , *rad51 Δ* , *mus81 Δ* and *pol32 Δ* cells exposed to CPT. Exponentially growing cells were
13 treated with DMSO or 50 μ M CPT for 2h and then pulse-labeled with 50 μ M EdU for 20min.
14 DNA fibers were combed on silanized coverslips and EdU-labeled DNA was detected by
15 Click chemistry. Graph depicts the distribution of EdU tracks length in kb. Box and whiskers
16 indicate 25-75 and 10-90 percentiles, respectively. Median EdU tracks length is indicated in
17 kb (values from two biological replicates were pooled). Asterisks indicate the *P*-value of the
18 Mann-Whitney unpaired t test, **: *p*=0.0015. The percentage of EdU track length decrease
19 between the DMSO and CPT conditions is indicated between parentheses for each strain.
20 Representative images of DNA fibers are shown. Red and white: EdU, green: DNA.

21 **(B)** Schematic representation of replication intermediates visualized by 2D gels.

22 **(C)** Analysis of replication intermediates by 2D gel electrophoresis. Replication intermediates
23 were monitored at early origin *ARS305* and region C in WT, *rad3-102*, *mus81 Δ* and *rad3-102*
24 *mus81 Δ* cells. Cells were synchronized in G₁ with α -factor and collected at the indicated time
25 points after release into S phase. A scheme of the studied chromosomal region is shown
26 (drawn to scale). Relevant probes are indicated by gray bars, and coordinates of ARS and
27 restriction sites are indicated in bp. Two biological replicates have been performed.

28 **Data information:** Accumulation of recombination molecules is indicated by white arrows.

29

30 **Figure 5.** Mus81 is involved in the processing of recombination intermediates in G₂/M after
31 replication restart.

32 **(A)** Analysis of nuclear Rad52-GFP foci formation. An illustrative image of the experimental
33 setup is shown. DIC, differential interference contrast; yellow, Rad52-GFP; red, mCHERRY-
34 Pus1 (nuclear compartment marker). Scale bar = 5 μ m.

1 **(B) (C)** Kinetic analysis of Rad52 foci formation. Wild-type and *mus81Δ* cells containing
2 Rad52-YFP and mCHERRY-Pus1 were synchronized in G₁ with α-factor, treated with
3 DMSO or 100 μM CPT, let in G₁ for 30 min, and released into S phase. Cells were collected
4 at the indicated time points and visualized by fluorescence microscopy. Mean ± SEM of cells
5 with Rad52 foci are shown for each time point. Flow cytometry profiles corresponding the
6 experimental setup are shown. **Four biological replicates have been performed.**

7 **(D)** Mms4-Flag10 phosphorylation analyzed by immunoblot in wild type cells exposed to
8 CPT. Wild-type cells were synchronized in G₁ with α-factor, treated with DMSO or 50 μM
9 CPT, let in G₁ for 1 h, and released into S phase. Cells were collected at the indicated time
10 points and Mms4 was immunodetected with anti-Flag antibodies. Clb2 immunodetection
11 serves as a marker for G₂ phase entry. FACS profiles corresponding the experimental setup
12 are also shown. **Two biological replicates have been performed.**

13 **(E)** CPT sensitivity assayed by 10-fold serial dilutions of *S-MUS81* and *G₂-MUS81* alleles
14 compared to the wild type, *mus81*, *mms4Δ* and *cdc5-2* mutants. Three biological replicates
15 have been performed.

16 **(F)** UV sensitivity assayed by 10-fold serial dilutions of *S-MUS81* and *G₂-MUS81* alleles in
17 combination with *rad3-102* on YPAD plates after exposure to the indicated UV-C doses.
18 **Three biological replicates have been performed.**

19

20 **Figure 6.** Recombination intermediates generated by replication restart accumulate in the
21 absence of Mus81.

22 **(A) (B)** Pulsed-field gel electrophoresis (PFGE) analysis of wild type, *mus81Δ*, *cdc5-2* and
23 *G₂-MUS81* cells in response to CPT. Cells were synchronized in G₁ with α-factor, treated
24 with DMSO or 100 μM CPT, let in G₁ for 1 h, and released into S phase. Cells were collected
25 at the indicated time points. DNA contents was analyzed by flow cytometry and the DNA
26 extracted in agarose plugs was analyzed by PFGE. Upper panel: agarose gel stained with
27 ethidium bromide; lower panel: Southern blot using a chromosome IV specific probe. JMs,
28 joint molecules accumulated in the gel wells. **The mean value of JMs relative to the total**
29 **amount of DNA is indicated for each time point. Two to three biological replicates have been**
30 **performed.**

31 **(C) (D)** PFGE analysis of *rad52Δ* and *rad52Δ mus81Δ* cells in response to CPT performed as
32 in (A) (B). The gel has been stained with Ethidium bromide and densitometry profiles
33 corresponding to the +CPT 140 min time points in *rad52Δ* and *rad52Δ mus81Δ* cells are
34 shown. **Two biological replicates have been performed.**

1

2 **Figure 7.** Proposed model for HR-mediated protection and restart of blocked replication
3 forks.

4 Replication fork block by CPT and in *rad3-102* cells would result in fork reversal, favoring
5 the removal of the replication impediment. The tip of the reversed fork would serve as a
6 substrate for the invasion of the parental duplex by HR, requiring Rad52, Rad51, the MRX
7 complex and the DNA polymerase δ (including Pol32). Replication could restart by BIR until
8 the merging of the D-loop with a convergent fork, producing a Holliday junction. Resolution
9 by Mus81 would promote replication termination. Mus81 could also cleave replication forks
10 that could not be restarted by HR (*e.g. rad52 Δ mutants*).

1 **EXPANDED VIEW FIGURE LEGENDS**

2

3 **Expanded View figure 1.** Yen1 backs up Mus81 in CPT-treated and *rad3-102* cells.

4 (A) CPT sensitivity assayed by 10-fold serial dilutions of different mutant combinations
5 between *mus81Δ* and *yen1Δ* on YPAD plates. Two biological replicates have been performed.

6 (B) UV sensitivity assayed by 10-fold serial dilutions of *yen1Δ* in combination with *rad3-102*
7 allele on YPAD plates after exposure to the indicated UV-C doses. Two biological replicates
8 have been performed.

9 (C) Synthetic combinations of *rad3-102 mus81Δ* with *yen1Δ* and *pol32Δ*. Tetrads dissected
10 on YPAD medium are shown. Triangles indicate lethality. Two biological replicates have
11 been performed.

12

13 **Expanded View figure 2.** DNA repair is similar in CPT-treated and *rad3-102* cells.

14 (A) Visualization of CPT-containing yeast cells. Exponentially growing wild type cells in
15 either MAD (minimal-ammonium-dextrose) or MPD +SDS (minimal-proline-dextrose +
16 0.003% SDS) medium were incubated with DMSO or 50 μM CPT for 30 min and visualized
17 by fluorescence microscopy. Scale bar = 5 μm. Three biological replicates have been
18 performed.

19 (B) Quantification of fluorescence emitted by CPT in cells grown as described in (A). Mean ±
20 SEM is indicated for each experimental condition. A.U. arbitrary units. Three biological
21 replicates have been performed.

22 (C) Analysis of DNA content by flow cytometry of G₁ phase synchronized wild type cells and
23 further released into S phase. Cells were synchronized in G₁ with α-factor in either MAD or
24 MPD +SDS medium, treated with DMSO or 50 μM CPT, let in G₁ for 30 min, and released
25 into S phase. Three biological replicates have been performed.

26 Asterisks indicate the progression of cells in S phase.

27

28 **Expanded View figure 3.** Mus81 is not required for replication fork progression in *rad3-102*
29 cells.

30 (A) Statistical analysis of the two biological replicates of DNA combing experiments,
31 corresponding to the data presented in Figure 4A. For each replicate, track length values of
32 DMSO-treated samples have been normalized to the median of wild type values. In each
33 mutant, track length values of CPT-treated samples have been converted using the same
34 normalization factor as for DMSO-treated samples. Graphs depict the distribution of

1 normalized EdU track length in kb. Box and whiskers indicate 25-75 and 10-90 percentiles,
2 respectively. Normalized median EdU tracks length is indicated in kb. Asterisks indicate the
3 *P*-value of the Mann-Whitney unpaired t test, **** *P*-value <0.0001, *** *P*-value <0.001, *
4 *P*-value =0.238, ns non-significant difference.

5 **(B)** Analysis of replicated DNA tracks length by single-molecule DNA combing in wild type,
6 *mus81Δ*, *rad3-102* and *rad3-102 mus81Δ* cells. Exponentially growing cells pulse-labeled
7 with 50 μM EdU for 20min. DNA fibers were combed on silanized coverslips and EdU-
8 labeled DNA was detected by Click chemistry. Graph depicts the distribution of EdU tracks
9 length in kb. Box and whiskers indicate 25-75 and 10-90 percentiles, respectively. Median
10 EdU tracks length is indicated in kb (values from two biological replicates were pooled).
11 Asterisks indicate the *P*-value of the Mann-Whitney unpaired t test, **** *P*-value <0.0001, **
12 *P*-value <0.01. Representative images of DNA fibers are shown. Red and white: EdU, green:
13 DNA.

14 **(C)** Analysis of DNA content by flow cytometry of cells collected for 2D gel analyses in
15 Figure 4C. Cells were synchronized in G₁ with α-factor in YPAD medium, released into S
16 phase and collected at the indicated time points. Two biological replicates have been
17 performed.

18 **(D)** Quantification of recombination intermediates (cone signal relative to Y arc signal) in two
19 independent experiments illustrated in Figure 4C. Individual and mean values are plotted for
20 each time point analyzed.

21

22 **Expanded View figure 4.** Mms4 phosphorylation in G₂/M is required for recombination
23 intermediates processing after replication restart.

24 **(A)** Analysis of Rad52 foci formation. Cells with Rad52-YFP foci were scored in
25 exponentially growing wild-type *mus81Δ*, *rad3-102* and *rad3-102 mus81Δ* cells. Mean and
26 SEM of cells with Rad52 foci for three biological replicates are shown. An illustrative image
27 is shown with Rad52 foci indicated by white arrows. Scale bar = 5 μm.

28 **(B)** Mms4-Flag10 phosphorylation analyzed by immunoblot in *rad52Δ* cells exposed to CPT.
29 *rad52Δ* cells were synchronized in G₁ with α-factor, treated with DMSO or 50 μM CPT, let in
30 G₁ for 1 h, and released into S phase. Cells were collected at the indicated time points and
31 Mms4 was immunodetected with anti-Flag antibodies. Clb2 immunodetection serves as a
32 marker for G₂ phase entry. Flow cytometry profiles corresponding the experimental setup are
33 shown. Two biological replicates have been performed.

1 (C) Mms4-Flag10 phosphorylation analyzed by immunoblot in wild type and *rad3-102* cells.
2 Cells were synchronized in G₁ with α -factor, untreated or irradiated with 30 J/m² UV-C, let in
3 G₁ for 2 h, and released into S phase. Cells were collected at the indicated time points and
4 Mms4 was immunodetected with anti-Flag antibodies. Clb2 immunodetection serves as a
5 marker for G₂ phase entry. Flow cytometry profiles corresponding the experimental setup are
6 shown. [Three biological replicates have been performed.](#)

7 (D) CPT sensitivity assayed by 10-fold serial dilutions of wild type and *mms4* mutants. [Three](#)
8 [biological replicates have been performed.](#)

9

10 **Expanded View figure 5.** DSBs are not detected in wild type CPT-treated and *rad3-102*
11 cells.

12 (A) Analysis of DNA content by flow cytometry of G₁ phase synchronized wild type and
13 *mus81* Δ cells and further released into S phase. Cells were synchronized in G₁ with α -factor,
14 treated with DMSO or 100 μ M CPT, let in G₁ for 1 h, and released into S phase. Asterisks
15 indicate when cells start the following cell cycle. [Three biological replicates have been](#)
16 [performed.](#)

17 (B) Pulsed-field gel electrophoresis (PFGE) analysis of wild type cells in response to CPT.
18 Wild type cells were synchronized in G₁ with α -factor, treated with DMSO or 200 μ M CPT,
19 let in G₁ for 1 h, and released into S phase. Cells were collected at the indicated time points.
20 DNA contents was analyzed by flow cytometry and the DNA extracted in agarose plugs was
21 analyzed by PFGE. The agarose gel has been stained with ethidium bromide. *zeo*, wild type
22 cells released from the G₁ arrest in the presence of 1 mg/mL Zeocin for 1 h. Zeocin-induced
23 DSBs are indicated by a vertical bar. [Three biological replicates have been performed.](#)

24 (C) PFGE analysis of wild type and *rad3-102* cells. Wild type and *rad3-102* cells were
25 synchronized in G₁ with α -factor, untreated or irradiated with 20 J/m² UV-C, let in G₁ for 2h,
26 and released into S phase. Cells were collected at the indicated time points. DNA contents
27 was analyzed by flow cytometry and the DNA extracted in agarose plugs was analyzed by
28 PFGE. Upper panel: agarose gel stained with ethidium bromide; lower panels: Southern blot
29 using a chromosome VIII specific probe. JMs, joint molecules accumulated in the gel wells.
30 [The mean value of JMs relative to the total amount of DNA is indicated for each time point.](#)
31 [Two biological replicates have been performed.](#)