Error-Free DNA Damage Tolerance and Sister Chromatid Proximity during DNA Replication Rely on the Polα/Primase/Ctf4 Complex

Highlights

- **Polα/Primase and cohesin support damage tolerance and sister chromatid proximity**
- **Artificial cohesion bypasses cohesin, but not Polα/Primase role in recombination**
- **Defects in Polα/Primase cause faulty strand annealing and reversed fork formation**
- **Altered ssDNA metabolism underlies Polα/Primase mutants damage tolerance defects**

In Brief

Fumasoni et al. explore the interplay between replication, sister chromatid cohesion, and recombination. Recombination and cohesion are facilitated by both cohesin and replication-fork-coupled re-priming. Cohesin does so by keeping the sister chromatids together, whereas replication-fork-coupled re-priming sustains normal fork architecture required for optimal cohesion and recombination-mediated DNA damage tolerance.
Error-Free DNA Damage Tolerance and Sister Chromatid Proximity during DNA Replication Rely on the Polα/Primase/Ctf4 Complex

Marco Fumasoni,1 Katharina Zwicky,2 Fabio Vanoli,1,3 Massimo Lopes,2 and Dana Branzei1,*

1IFOM, the FIRC Institute of Molecular Oncology, Via Adamello 16, 20139 Milan, Italy
2Institute of Molecular Cancer Research, University of Zurich, CH-8057, Zurich, Switzerland
3Present address: Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
*Correspondence: dana.branzei@ifom.eu
http://dx.doi.org/10.1016/j.molcel.2014.12.038
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

Chromosomal replication is entwined with DNA damage tolerance (DDT) and chromatin structure establishment via elusive mechanisms. Here we examined how specific replication conditions affecting replisome architecture and repriming impact on DDT. We show that Saccharomyces cerevisiae Polα/Primase/Ctf4 mutants, proficient in bulk DNA replication, are defective in recombination-mediated damage-bypass by template switching (TS) and have reduced sister chromatin cohesion. The decrease in error-free DDT is accompanied by increased usage of mutagenic DDT, fork reversal, and higher rates of genome rearrangements mediated by faulty strand annealing. Notably, the DDT defects of Polα/Primase/Ctf4 mutants are not the consequence of increased sister chromatid distance, but are instead caused by altered single-stranded DNA metabolism and abnormal replication fork topology. We propose that error-free TS is driven by timely replicative helicase-coupled re-priming. Defects in this event impact on replication fork architecture and sister chromatid proximity, and represent a frequent source of chromosome lesions upon replication dysfunctions.

INTRODUCTION

Faithful DNA replication is crucial for genomic maintenance. When replication is perturbed, cells activate stress response networks that connect the detection of replication-blocking lesions with DNA damage tolerance (DDT) and repair pathways, chromatin modifications, cell-cycle control, and various other changes in cell physiology, often collectively referred to as the DNA damage response (DDR) (Jackson and Bartek, 2009). Failures in these processes are implicated in the etiology of many developmental and neurological disorders and are thought to drive genome instability characteristic of cancer (O’Driscol and Jeggo, 2008).

Genome duplication is carried out by the replisome machinery, initially assembled at replication origins (Gerbi and Bielinsky, 2002). Notably, replication initiation critically depends on the loading and activity of the Polymerase α (Polα)/Primase complex. This is the fundamental initiator of DNA replication in eukaryotic cells, as the replicative DNA polymerases can only elongate an existing RNA-DNA primer produced by this complex. The Primase produces short RNA fragments (about 7–12 nt long), which are subsequently subjected to limited extension by Polz. These RNA-DNA primers are then extended by the replicative polymerases Polz and Polα (Aze et al., 2013).

Polα/Primase-mediated processes are not only relevant for origin-dependent replication initiation, but also for origin-independent initiation events, as is the case of lagging strand DNA synthesis, and possibly the restart of stalled forks downstream the blocking lesion under conditions of genotoxic stress (Branzei and Foiani, 2010; Heller and Marians, 2006). The latter aspect is potentially crucial for efficient DDT and replication, especially in conditions in which fast replication is a requirement, such as at the early stages of development (O’Driscoll and Jeggo, 2008).

Two distinct modes of DDT, error-prone and error-free DDT, operate in all eukaryotic organisms (Sale, 2012). Error-prone DDT is mediated by translesion synthesis (TLS) polymerases and largely accounts for mutagenesis. Error-free DDT uses a recombination-related mechanism known as template switching (TS), in which one newly synthesized strand serves as replication template for the other blocked nascent strand (Branzei, 2011; Giannattasio et al., 2014). The choice between these DDT modes has profound consequences for genome stability, and to date, several factors have been implicated in DDT pathway choice: PCNA post-translational modifications with mono-ubiquitylation, poly-ubiquitylation, and SUMOylation (Branzei et al., 2008; Hoege et al., 2002; Papouli et al., 2005; Pfander et al., 2005); genome architectural transitions coupled with early stages of replication (Gonzalez-Huici et al., 2014); and cell-cycle-specific changes in the abundance or regulation of key DDT factors (Karras et al., 2013; Waters and Walker, 2006).

Together with Polα/Primase, a number of structural proteins that tether the replicative minichromosome maintenance (MCM) helicase to the replicative polymerases are loaded at replication origins (Aze et al., 2013). Ctf4 (AND-1 in mammalian...
cells) functions as such a replisome architectural factor, bridging the MCM helicase and two molecules of Polα/Primase (Gambus et al., 2009; Simon et al., 2014). It is of note, however, that while Polα and Primase are essential for cellular proliferation, Ctf4 is not. This indicates that even if uncoupled from the replicative helicase, Polα/Primase supports DNA synthesis. Besides its roles to maintain normal replisome architecture, Ctf4/AND-1 is also required for sister chromatid cohesion (Hanna et al., 2001; Yoshizawa-Sugata and Masai, 2009).

Increasing number of reports indicate “replication stress” at the basis of chromosomal instability, and as an important underlying factor of developmental anomalies (Halazonetis et al., 2008; Jackson and Bartek, 2009). However, the nature of the early chromosome lesions arising following such replication perturbations is largely unknown. Moreover, the connections between these replication dysfunctions and the observed chromatin structural alterations similarly triggered by mutations in cohesion factors remain elusive.

Here we used budding yeast Saccharomyces cerevisiae cellular models of specific replication stress and sister chromatid cohesion defects to investigate a possible crosstalk between recombination-mediated DDT and chromatin structure/cohesion. Our results indicate that both replicative helicase-coupled re-priming and sister chromatid cohesion are important to facilitate error-free DDT by TS, but they do so via different mechanisms. The results shed light on how highly conserved replication-associated pathways crosstalk to each other and contribute to normal replication fork and chromatin structure, providing mechanistic insights into the molecular basis of human disorders caused by replication dysfunctions.

RESULTS

Ctf4 Promotes Template Switching Independently of Canonical Error-Free DDT Pathways

Error-free DDT by TS can be molecularly monitored by examining the formation of X-shaped structures composed of sister chromatid junctions (SCJs) in the proximity of replication forks using 2D gel electrophoresis (Branzei et al., 2008). In this assay, yeast cells are released synchronously and allowed to replicate in media containing the alkylating reagent methyl methanesulfonate (MMS). The pattern of replication intermediates at genomic locations of interest is analyzed at different time points during DNA replication. Previous results showed that TS intermediates form during replication of damaged templates and accumulate when the Sgs1-Top3-Rmi1 complex is defective because of compromised resolution (Branzei et al., 2008; Liberi et al., 2005; Giannattasio et al., 2014). As a consequence, replication of damage templates leads to a progressive accumulation of SCJs in sgs1Δ mutant cells (Liberi et al., 2005), as well as in sgs1 hypomorphic mutants disrupted only in the helicase domain (Onoda et al., 2000; Vanoli et al., 2010) (Figure 1A).

Deletion of CTF4 causes a G2/M delay associated with slower proliferation (Kouprina et al., 1992), but it does not prominently impact on S phase progression when cells are replicating in the presence of MMS (Figure S1A). Notably, we found that the ctf4Δ mutation reduced the amount of X-shaped SCJs in both sgs1 and wild-type (WT) background (Figures 1A and S1B), suggesting a supportive role for Ctf4 in TS.

Cells defective in error-free DDT are characterized by a more prominent dependency on TLS, showing higher levels of spontaneous mutagenesis and increased damage sensitivity following inactivation of error-prone DDT components (Cejka et al., 2001). We found that ctf4Δ cells have statistically significant increased spontaneous mutagenesis rates (Figure 1B). This was largely dependent on REV3 (Figure 1B)—the catalytic subunit of the TLS polymerase Polζ, accounting for most of the mutagenic events in budding yeast. Furthermore, ctf4Δ and rev3Δ showed strong additive effects for MMS sensitivity (Figure S1C), supporting the view that TLS-mediated DDT acts as a compensatory mechanism in ctf4Δ cells.

To further characterize the role of Ctf4 in DDT, we examined its genetic interactions with classical error-free DDT pathways governed by RAD6-RAD18 post-replicative repair and RAD51 recombinational repair branches. ctf4Δ mutants showed additive damage sensitivity when combined with rad18Δ or rad51Δ (Figure S1D). Since the RAD6-RAD18 pathway controls both TS- and TLS-mediated DDT, we also examined the genetic interaction of CTF4 with the PCNA polyubiquitylation pathway (mediated by RAD5, MMS2, and UBC13) that specifically affects TS (Pfander et al., 2005). However, also in this case, we found an additive effect on damage survival (Figure S1E). Next, to address the possibility of an effect of Ctf4 on DDT by influencing the post-translational modifications of PCNA, we analyzed the pattern of SUMO- and ubiquitin/polyubiquitin-conjugated PCNA species in ctf4Δ cells. We detected no major changes in PCNA modifications both in spontaneous and MMS-treated conditions (Figure 1C). We conclude that Ctf4 facilitates error-free DDT in parallel with canonical post-replicative repair pathways regulated by PCNA modifications with SUMO and ubiquitin.

Polα/Primase and Cohesin Facilitate Error-Free DDT and Mediate Sister Chromatid Cohesion

Previous work assigned two main functions to Ctf4: an architectural role in the context of the replisome—achieved by bridging the Polα/Primase complex to the MCM replicative helicase (Gambus et al., 2009; Simon et al., 2014; Tanaka et al., 2009)—and an additional role in sister chromatid cohesion (Hanna et al., 2001). A CTF4 deletion mutant shows a mild increase in premature sister chromatid separation in G2/M, accompanied by sporadic chromosome loss and aneuploidy without obvious defects in completing the bulk of DNA replication (Hanna et al., 2001; Kouprina et al., 1992). It is therefore possible that the TS defect of CTF4 mutants (Figure 1A) is an indirect consequence of defective sister chromatid cohesion or, alternatively, a direct result of MCM-uncoupled Polα/Primase compromised activity.

The first hypothesis leads to the proposition that mutants defective in cohesion, but proficient for Polα/Primase function, would also be defective in formation of TS intermediates composed of SCJs. To test this, we analyzed the consequences for TS of impairing sister chromatid cohesion using genetic conditions that result in loosening or opening of the cohesin ring.
Scc1 is an essential subunit of the cohesin complex that is cleaved at anaphase (Uhlmann et al., 1999). Using the temperature-sensitive allele, scc1-73, we found that impairment of cohesin function during a single round of replication also caused a defect in TS (Figures 2A). Similar results were obtained using a GAL promoter-driven conditional SCC1 allele, GAL-SCC1, the expression of which is induced by galactose and repressed by glucose (data not shown).

Next, we asked if Polα/Primase mutants are different or similar to Ctf4 and cohesin mutants with respect to TS. We investigated the contribution of two alleles, pri1-M4, affecting the Pri1 subunit of Primase (Marini et al., 1997), and pol1-1, affecting the catalytic subunit Pol1 of Polα (Lucchini et al., 1988). To mildly interfere with the function of Polα/Primase without affecting the ability of cells to traverse S phase, cells were grown at the permissive temperature and synchronously released in media containing MMS at semi-permissive temperatures that allow completion of DNA replication with normal kinetics (data not shown; see below). Both pri1-M4 and pol1-1 mutations caused a reduction in the X molecules accumulating in sgs1 (Figures 2B and S2A). This phenotype is suggestive of a positive role of Polα/Primase in error-free DDT by TS, a conclusion also supported by the increased mutagenesis in Polα and we examined the percentage of premature sister chromatid separation, a widely used cohesion readout, in pri1-M4 cells. Compared to WT, pri1-M4 cells exhibited a significant increase in premature sister chromatid separation, at a level similar with the one caused by mutations in the non-essential cohesion factors Ctf4, Chl1, and Ctf18 included in the analysis (Figure 2C). Previously, based on genetic interactions, non-essential cohesion factors were divided in two genetic pathways, having as prominent members Ctf4 and Chl1 or Ctf18 and Ctf8, respectively (Xu et al., 2007). While combination of mutations in the same cohesion pathway generally does not exacerbate the observed cohesion defects associated with single mutations and does not lead to synthetic fitness defects, the opposite is true for combinations of mutations belonging to separate pathways. To further test if the cohesion defects observed with pri1-M4 (Figure 2C) are manifested in the context of the Polα/Primase/Ctf4 complex, we combined pri1-M4 with deletion mutations in genes affecting the two non-essential cohesion pathways. Supporting the above view that pri1-M4 cohesion defects reflect a role for Polα/Primase/Ctf4 in cohesion, pri1-M4 manifested synthetic fitness defects with ctf18Δ and ctf8Δ, but not with ctf4Δ or chl1Δ (Figure S2B; data not shown).

Figure 1. Ctf4 Facilitates Error-Free DDT

(A) sgs1 (HY1461) and sgs1 ctf4Δ (HY1472) cells were synchronized in G1 with alpha-factor (α) at 25°C prior to release at 30°C in media containing 0.033% MMS. Genomic DNA, extracted from samples collected at the indicated time points, was digested with NcoI and analyzed by 2D gel with a probe for ARS305. Schematic representation of major 2D gel signals, FACS, and X molecule quantification plots are displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges.

(B) Spontaneous mutations rates at CAN1 locus (x10⁻⁷) in WT (FY0001), rev3Δ (HY0008), ctf4Δ (HY3466), and ctf4Δ rev3Δ (HY3468). Mutation rates and 95% confidence intervals were estimated using the maximum-likelihood method. Non-overlapping confidence intervals indicate statistical significance.

(C) WT (FY1000) and ctf4Δ (HY2193) cells were grown at 25°C and then shifted to 30°C for 2 hr in YPD or YPD containing 0.02% MMS. PCNA modifications were detected using a monoclonal antibody against PCNA. Ponceau staining serves as loading control. See also Figure S1.

Pri1 mutants previously reported (Longhese et al., 1993; Suzuki et al., 2009).

Considering the phenotypic similarity between ctf4Δ and both cohesin and Polα/Primase mutants with regard to TS, we asked if Ctf4 contribution to cohesion might take place in the context of the Polα/Primase/Ctf4 complex. To this purpose,
Altogether, these data demonstrate that interfering with the functionality of the Polα/Primase/Ctf4 and cohesin complexes results in similar defects both in cohesion and error-free DDT by TS, leading to the question of whether cohesion and TS recombination are linked by a causal relationship.

**Cohesin and Polα/Primase/Ctf4 Complexes Promote TS by Distinct Molecular Mechanisms**

Sister chromatid cohesion defects reflect impairments in maintaining accurate physical proximity between the newly replicated chromatids. While it is reasonable to assume that by influencing
the accessibility of the sister chromatid donor sequence cohesin facilitates sister chromatid recombination (Covo et al., 2010; Tittlemier et al., 2012), to what extent the physical proximity of chromatids impacts on SCJ formation during the non-canonical recombination mechanism of TS is unknown. To address this, we used a previously described system that artificially re-establishes cohesion in a locus-specific manner. The system relies on the ability of the Lactose inhibitor (LacI) to bind the Lactose operator sequence (LacO). In the WT form (Tetramer; Figure 3A, right), LacI is able to form tetramers that bind two different LacO sequences present on each chromatid, while a truncated version of the LacI protein (Dimer; Figure 3A, left) is only able to form a dimer and to bind the LacO sequences on a single chromatid. Therefore, after DNA replication, only the tetramer version of LacI, and not the dimer, can bind the identical LacO sequences placed on both sister chromatids, restoring sister chromatid cohesion locally in cohesion-defective mutants (Straight et al., 1996).

By using the above assay, we examined whether artificial tethering of the sister chromatids was able to locally rescue the TS defects associated with cohesin mutants. sgs1Δ and sgs1Δ scc1-73 cells carrying either the dimer or the tetramer LacO/LacI system were synchronized at the permissive temperature, and after activation of LacI expression, cells were released in media containing MMS at the non-permissive temperature for scc1-73 to induce TS defects associated with decreased cohesion. Therefore, after DNA replication, only the tetramer version of LacI, and not the dimer, can bind the identical LacO sequences placed on both sister chromatids, restoring sister chromatid cohesion locally in cohesion-defective mutants (Straight et al., 1996).

Figure 3. Artificial Tethering of Sister Chromatids Suppresses the Template Switching Defects of Cohesin Mutants
(A) Schematic representation of the dimeric and tetrameric LacI systems for sister chromatid tethering.
(B) sgs1Δ and sgs1Δ scc1-73 cells carrying dimeric LacI (HY4252 and HY4255) or tetrameric LacI (HY4259 and HY4262) were grown at 25°C and synchronized in G2 by nocodazole (N) treatment. Cells were transferred to synthetic complete media lacking histidine and supplemented with 10 mM 3-aminotriazole and nocodazole for the last 20 min of the arrest to induce LacI expression. Cells were then released at 37°C in media containing 0.033% MMS, and samples were taken at the indicated time points. The genomic DNA was digested with EcoRV/XhoI and analyzed with a probe flanking the LEU2 locus. FACS and X molecule quantification plots are displayed. The columns in the quantification graphs denote the data mean of two independent experiments, and the bars indicate ranges. See also Figure S3.
cohesin function (see Figure 2A). Next, we examined by 2D gel the pattern of TS intermediates arising at locations proximal to the LacO array (Figure 2A). We found that, unlike the sgs1\textsuperscript{D} scc1-73 mutant carrying the dimer version of LacI, which showed reduced accumulation of TS intermediates (Figure 3B, upper panel), the strain with the tetramer version of LacI rescued X molecule formation (Figure 3B, bottom panel). To verify the specificity of the tethering system, we digested and hybridized the same genomic DNA to probe for replication intermediates forming in the proximity of the early origin of replication ARS1, located on chromosome IV, which should not be affected by the LacI variants binding to LacO cassettes on chromosome III. As expected, the TS defect associated with scc1-73 at ARS1 was not alleviated (Figure S3). Because artificial tethering of the chromatids is able to bypass the absence of a functional cohesin ring in regard to SCJ formation, these experiments demonstrate that the role exerted by the cohesin complex in TS is structural.

To now assess whether, as in scc1-73, the ctf4\textsuperscript{D} defect in TS is also due to increased physical distance between the sister chromatids, we used the same experimental approach, but in the sgs1 ctf4\textsuperscript{D} background. Interestingly, artificial cohesion did not restore the formation of X molecules in the sgs1 ctf4\textsuperscript{D} strain (Figure 4A), although the LacO/LacI system efficiently reduced the local cohesion defects on chromosome III associated with ctf4\textsuperscript{D} (Figure 4B). The function of Ctf4 in TS revealed by this assay is thus different from the structural one of cohesin (Figure 3B). Of note, the defect of ctf4\textsuperscript{D} cells

![Figure 4. Artificial Cohesion Does Not Restore Efficient Template Switching in ctf4\textsuperscript{D} Mutants](image)

(A) sgs1 and sgs1 ctf4\textsuperscript{D} cells carrying dimeric LacI (HY3979 and HY3981) or tetrameric LacI (HY3983 and HY3985) were grown and arrested as in Figure 3B. Cells were then released at 30°C in media containing 0.033% MMS. 2D gel analysis was conducted as in the Figure 3B. FACS and X molecule quantification plots are displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges.

(B) Premature sister chromatid separation in sgs1 ctf4\textsuperscript{D} cells carrying the dimeric and tetrameric version of the LacI. The experiment was conducted as in Figure 2C. See also Figure S4.
resembled the one caused by inactivation of the Rad51 recombinase (Figure S4A). These results indicate that Ctf4 affects key aspects of the TS reaction that cannot be substituted by simply restoring the physical proximity of the sister chromatids. Taken together, the data suggest that the repair/TS defect of ctf4Δ mutants is primarily caused by faulty MCM-uncoupled Polα/Primase activity rather than by defective sister chromatid cohesion.

Based on the above results, we conclude that, at least in regard to DDT, the roles of cohesin and Polα/Primase/Ctf4 are manifested via two fundamentally distinct mechanisms. Supporting this view, deletion of CTF4 resulted in synthetic lethality when combined with scc1-73 (Figure S4B) (McLellan et al., 2012). In addition, using a combination of temperature-sensitive alleles of Polα/Primase mutants and cohesin, we obtained viable pri1-M4 scc1-73 cells that displayed, however, increased MMS sensitivity compared to the single mutants (Figure S4C). This result highlights once again a differential contribution of cohesin and the Polα/Primase/Ctf4 complex toward DDT. A diverse molecular purpose of Polα/Primase and cohesin during canonical recombination can also be deduced from their contribution to the donor choice: in cohesin mutants, the inter-sister recombination events decrease in favor of events involving the homologous chromosome (Covo et al., 2010; Tittel-Elmer et al., 2012), whereas ctf4Δ causes a decrease in both sister- and inter-homologous recombination in conditions of DNA damage (Ogiwara et al., 2007).

Altogether, these results reveal that, despite cohesin and ctf4Δ mutants showing similar phenotypes in cohesion and DDT, cohesin aids TS by maintaining the proximity of the recombination donor, while the Polα/Primase/Ctf4 complex must affect some fundamental activity or step during recombination-mediated damage bypass.

**A Defective Polα/Primase/Ctf4 Complex Causes Unscheduled Strand Annealing and Fork Reversal**

To identify the TS-sensitive step that is defective in Polα/Primase/Ctf4 complex mutants, when re-priming is either affected or uncoupled from the MCM helicase, we combined ctf4Δ and pri1-M4 with a series of mutations affecting distinct HR-associated processes. We found that ctf4Δ shows synthetic sickness/lethality with rad52Δ (Kouprina et al., 1992), but not with rad51Δ (Figure 5A) or other Rad51 mediators, such as Rad55, that assist Rad51-mediated strand invasion (data not shown). We also observed a similar pattern of genetic interactions for pri1-M4 (Figure S5A).

Although Rad51 and Rad52 act together in most recombination events, a significant amount of HR events are detectable in rad51Δ mutants that are dependent on Rad52 (Krogh and Symington, 2004). In Saccharomyces cerevisiae, the major Rad51-independent activity of Rad52 is represented by strand annealing, which also involves Rad59 and RPA (Krogh and
Notably, ctf4Δ also showed synthetic fitness defects when combined with rad59Δ (Figure 5B) and the S. cerevisiae RPA largest subunit RFA1 allele, rfa1-T11 (Figures 5C; see below). However, no synthetic interactions were observed with rad1Δ (Figure S5B), defective in the process of double-strand break (DSB) repair by single-strand annealing (Krogh and Symington, 2004).

The single-stranded DNA-binding complex RPA initially covers ssDNA in order to prevent secondary structure formation and to mediate the subsequent binding of Rad52 (Krogh and Symington, 2004). Binding of Rad52 to stretches of RPA-covered ssDNA may promote annealing of such sequences, potentially leading to rearrangements, such as deletions and duplications, especially in the presence of repeat sequences (Branzei, 2011).

Supporting this view, we found a strong increase, of a 1,000-fold magnitude, in spontaneous deletion rates of genomic elements flanked by repeat sequences, in both ctf4Δ and pri1-M4 mutants with respect to WT (Figure 5D). These deletion events were not dependent on Rad51; rather, the rad51Δ mutation itself caused an increase in such deletion events in both WT and ctf4Δ/pri1-M4 backgrounds (Figure 5D).

To directly test the genetic evidence of an altered amount of ssDNA in ctf4Δ and pri1-M4 mutants, we performed electron microscopy (EM) analysis on the replication intermediates extracted from WT, ctf4Δ, and pri1-M4 cells replicating under damaging conditions. pri1-M4 showed a strong increase in the length of the ssDNA stretches exposed at the replication fork (Figure 6A). In addition to this, we found that although not different for the average length, both ctf4Δ and pri1-M4 mutants showed a 2-fold increase in the number of internal gaps detected behind replication forks (Figure 6B).

Defective re-priming may lead to replication fork rearrangements. Interestingly, we observed reversed forks—Holliday Junction-like structures in which the two newly synthesized strands are paired, in both ctf4Δ and pri1-M4 (Figure 6C). It is of note that such reversed fork structures were absent in WT cells, suggesting that reversed forks are not predominantly
induced following genotoxic stress in Saccharomyces cerevisiae. This is in line with previous reports on total replication intermediates formed during replication in the presence of UV- or MMS-induced damage (Lopes et al., 2006), and with the structural analysis of X-shaped structures forming at a specific chromosome locus during replication of damaged templates in WT and sgs1 budding yeast cells (Giannattasio et al., 2014).

Altogether, the results demonstrate a profound alteration in the metabolism of ssDNA in Polα/Primase/Ctf4 complex mutants. This likely leads to the subsequent formation of unscheduled reversed forks and recombination structures, having negative effects on both chromatin structure and DDR.

**DISCUSSION**

Rapid cell proliferation is required especially at the early stages of development. This process generates endogenous damage and replication stress, although the molecular nature of the latter remains poorly understood. While various studies outline that intimate links must exist between DNA replication and DDR mechanisms (Branzei and Foiani, 2010; Jackson and Bartek, 2009), how relatively mild replication-challenging conditions interfere with other chromosome metabolism processes associated with DNA replication remains largely elusive.

Here we set out to examine how specific replication pathways may crosstalk to each other and affect DDR. Remarkably, we found that mild impairment in replication-associated molecular pathways related to re-priming, replisome architecture, and cohesion invariably negatively impinged on template switching of genomic rearrangements (Figures 1B and 5D), as well as with altered replication fork structures (Figure 6). These results provide in vivo support for the primer-driven post-replicative DDR as the most prominent pathway of DDR, at least in budding yeast (Becker et al., 2014; Branzei, 2011; Heller and Marians, 2006; Lehmann and Fuchs, 2006). Moreover, as deletion of CTF4 that does not directly impact on Primase activity caused a similar phenotype to the one of Primase mutants, the present work carries the implication that this re-priming function provides genome stability when carried out in the context of MCM-coupled Polα/Primase protein complex (Figure 7). On the contrary, when re-priming is deregulated, problems are bound to occur. Limited re-priming can lead to fork uncoupling and long stretches of ssDNA at the fork (Figure 6A); these ssDNA discontinuities can engage in error-prone annealing events (Figure 5D) and cause altered replication fork architecture (Figures 6C and 7). Deregulated re-priming events can lead to a larger number of internal gaps (Figure 6B), some of which may be more easily filled-in via TLS-mediated mechanisms (Figure 1B) or also engaged by error-prone strand-annealing activities (Figures 5D and 7).

Notably, under conditions of limited or MCM-uncoupled re-priming, we observe an unusual high frequency of fork-reversal events associated with long ssDNA stretches at the fork. The ssDNA regions may be due to prolonged replication fork uncoupling or exonucleolytic processing of the reversed forks (Figure 7). These observations indicate that—at least in Saccharomyces cerevisiae—fork reversal is disfavored or extremely transient when re-priming is working efficiently. The fork reversal

![Figure 7. Hypothetical Model of DDR Orchestration in WT Cells versus Primase/Ctf4 Complex Mutants](https://example.com/figure7.jpg)

Response of replication forks encountering DNA damage; parental DNA is shown in black, newly synthesized chromatids in blue, the DNA lesion is represented by a white star, and the RNA-DNA primer synthesized by Polα/Primase in orange. In WT cells, physiological repriming activity allows restart of replication downstream the lesion and then post-replicative tolerance of the damage by template switching. In Ctf4 and Primase mutants, defects in synthesizing a new primer generate uncoupling of the leading and lagging strands with the formation of longer ssDNA stretches at the fork. The exposure of ssDNA promotes the annealing of homologous sequences that may result in spontaneous deletion events and fork reversal. Reversed forks can be further processed or mediate replication fork restart by BIR or other annealing-mediated events. In addition, the Polα/Primase/Ctf4-defective complex may perform unscheduled repriming attempts that would result in an increase of internal ssDNA gaps that can be filled-in partially by template switching or TLS events.

(Figures 1A and 2B). The defects in error-free DDR were coincident with an increase in mutagenic DDR and other types of genomic rearrangements (Figures 1B and 5D), as well as with altered replication fork structures (Figure 6). These results provide in vivo support for the primer-driven post-replicative DDR as the most prominent pathway of DDR, at least in budding yeast (Becker et al., 2014; Branzei, 2011; Heller and Marians, 2006; Lehmann and Fuchs, 2006). Moreover, as deletion of CTF4 that does not directly impact on Primase activity caused a similar phenotype to the one of Primase mutants, the present work carries the implication that this re-priming function provides genome stability when carried out in the context of MCM-coupled Polα/Primase protein complex (Figure 7). On the contrary, when re-priming is deregulated, problems are bound to occur. Limited re-priming can lead to fork uncoupling and long stretches of ssDNA at the fork (Figure 6A); these ssDNA discontinuities can engage in error-prone annealing events (Figure 5D) and cause altered replication fork architecture (Figures 6C and 7). Deregulated re-priming events can lead to a larger number of internal gaps (Figure 6B), some of which may be more easily filled-in via TLS-mediated mechanisms (Figure 1B) or also engaged by error-prone strand-annealing activities (Figures 5D and 7).

Notably, under conditions of limited or MCM-uncoupled re-priming, we observe an unusual high frequency of fork-reversal events associated with long ssDNA stretches at the fork. The ssDNA regions may be due to prolonged replication fork uncoupling or exonucleolytic processing of the reversed forks (Figure 7). These observations indicate that—at least in Saccharomyces cerevisiae—fork reversal is disfavored or extremely transient when re-priming is working efficiently. The fork reversal
phenotype we reported here for ctf4Δ and pri1-M4 mutants is induced by DNA damage. While in Primase mutants about 12% of the forks are present in reversed conformation during replication in the presence of MMS (Figure 6C), following fork stalling induced by HU treatment, we found only 1% reversed forks in Primase mutants and no reversed forks in WT and ctf4Δ mutants (data not shown). This is in contrast with the phenotype of rad53 replication checkpoint mutants in which about 10% of replication forks are in a reversed fork conformation after HU treatment (Cotta-Ramusino et al., 2005; Sogo et al., 2002). Accumulation of reversed forks upon checkpoint defects likely reflects local accumulation of torsional stress (Bermúdez et al., 2011) and not DDT attempts, as only about 1% reversed forks are observed in checkpoint defective cells upon MMS treatment (Lopes et al., 2006). Thus, replication fork reversal is differentially modulated upon challenges to replisome stability or during replication-associated DDT. Under specific types of genotoxic stress (Ray Chaudhuri et al., 2012), or upon impairment of kinetically favored DDT mechanisms (e.g., repriming), transient fork reversal may become a crucial strategy to promote fork stabilization or to mediate alternate modes of damage-bypass in both S. cerevisiae and higher eukaryotic cells (Neelsen and Lopes, 2015).

While fork reversal may initially function as a fork stabilization mechanism both at stalled and damaged replication forks (Atkinson and McGlynn, 2009; Neelsen and Lopes, 2015), it is of note that—unless promptly restarted—reversed forks can be processed by multiple nucleases (Cotta-Ramusino et al., 2005; Doksan et al., 2009; Neelsen et al., 2013; Szakal and Branzoi, 2013), thus being a potential source of deleterious ssDNA discontinuities and DSBs that may trigger, in the long run, genome instability (Figure 7). Indeed, reduced amounts of Polα in Saccharomyces cerevisiae induce recombination-associated chromosome deletions and duplications (Song et al., 2014). We propose that altered fork structures may be engaged in break-induced replication (BIR) type of mechanisms (Figure 7), which may function as alternate and likely aberrant modes of DDT, prone to deletions as well as genomic duplications (Costantino et al., 2014).

Notably, in support of the BIR notion above, we found that ctf4Δ and pri1-M4 mutants display synthetic lethality with deletions of RADS2 and RADS9, generally required for BIR, but not with RADS1, which is often dispensable for BIR (Anand et al., 2013). As many replication factors, including Ctf4, also facilitate the DNA synthesis step of BIR (Lydeard et al., 2010), it is likely that the BIR events induced under such replication stress conditions might be more prone to errors, leading to rearrangements. Indeed, inactivating mutations in error-free DDT factors as well as in Polα/Primase cause an increase in genomic duplications and deletions (Figure 5D) (Putnam et al., 2010). Altogether, these results provide a molecular rationale for why cellular conditions characterized by replication stress are coincidently defective in error-free DDT processes and prone to deleterious BIR or other annealing events (Figures 1 and 5). These recombination pathways are bound to involve similarly defective replication steps, thus setting the stage for vicious circles in which more replication stress is being created.

Mild replication dysfunctions of the type we uncovered here in ctf4Δ and pri1-M4 mutants are likely the culprits not only for genome instability, but also for chromosomal structural and cohesion alterations. Indeed, we found that differently from impairments in cohesin, the cohesion and Ts defects of Polα/Primase/Ctf4 are in a non-causal relationship. On one hand, improvement of sister chromatid proximity does not rescue the Ts defects of ctf4Δ mutants (Figure 4). On the other hand, inability to perform TS is unlikely at the basis of the observed cohesion defects, as mutations in canonical TS factors do not result in similar cohesion defects (Warren et al., 2004). We propose that the two phenotypes, defective DDT and cohesion impairment, arise coincidentally, but likely independently, from the same replication condition. In the case of Polα/Primase/Ctf4 mutants, the underlying replication dysfunction relates to deregulated re-priming, and abnormal distribution of ssDNA discontinuities. This may impact on the loading or residence time of cohesin on chromatin. In addition, it is possible that increased replication pausing and altered fork structure in Polα/Primase/Ctf4 complex mutants affects the topology of the replicating DNA in manners that negatively impinge on cohesion (Liu et al., 2010).

The coincident cohesion and DDT defects associated with mutations in the Polα/Primase/Ctf4 complex suggest an intimate relationship between these two biological processes during replication. This hypothesis may also explain that hypomorphic mutations in other replication factors, affecting replication initiation or primer-processing, lead to cohesion defects and reduced tolerance to genotoxic stress (Kerzendorfer et al., 2013; van der Lelij et al., 2010). Remarkably, oncogene-induced replication stress, as well as DDR and cohesion dysfunctions associated with neurodevelopmental defects, resemble in many respects the replication impairments studied here (Halazonetis et al., 2008; Neelsen et al., 2013; O’Driscoll and Jeggo, 2008; van der Lelij et al., 2010). We propose that aberrant DDT associated with compromised error-free TS and fork rearrangements is a potential underlying common source of lesions in a number of replication disorders.

EXPERIMENTAL PROCEDURES

The experiments were conducted as described in the figure legends. Detailed procedures are given in the Supplemental Experimental Procedures.

Yeast Techniques

The yeast strains used in this study were mostly derivatives of W303 and detailed genotypes are indicated in Table S1. Information about growing media, cell cycle arrest, release, and analysis are described in Supplemental Experimental Procedures.

Mutation and Deletion Rates

Forward mutation and spontaneous intra-chromosomal deletion rates were calculated as described in the Supplemental Experimental Procedures.

Extraction of Replication Intermediates and 2D Gel Procedure

Purification of DNA intermediates and 2D gel analysis were carried out as previously described (Vanoli et al., 2010). Each experiment shown was performed independently at least twice with qualitatively identical results. Genome preparation and signal quantification methods are described in the Supplemental
Experimental Procedures. Restriction enzyme digestion strategies and DNA probes used are indicated in the figure legends.

Replication Intermediates Enrichment and EM Analysis
Enrichment of the replication intermediates was performed as in Neelsen et al. (2014) and as described in the Supplemental Experimental Procedures.

Premature Sister Chromatid Separation Assay
Sister chromatid cohesion was measured as previously described (Michaelis et al., 1997) and detailed in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.12.038.

ACKNOWLEDGMENTS
We thank M. Foiani for the pri1-M4 allele, A. Straight and A.W. Murray for the LacO/LacI system, K. Nasmyth for assistance with the EM analysis; I. Psakhye and T. Abe for critical reading of the manuscript; M. Giannattasio, D. Menolfi, and B. Szakal for experimental help; and the Branzei lab members for various discussions. This work was supported by the Swiss National Science Foundation grant 31003A_146924 to K.Z. and M.L., and Fondazione Telethon (GGP12160), AIRC (IG 14171) and ERC (Starting Grant 242928) grants to D.B. M.F. was partially supported by a FIRC fellowship (Mario e Valeria Rindi).

Received: August 27, 2014
Revised: November 15, 2014
Accepted: December 22, 2014
Published: February 5, 2015

REFERENCES

Andersson, S., Reis, U., and Haber, J.E. (2013). Break-induced DNA replication. Cold Spring Harb. Perspect. Biol. 5, a010397.

Atkinson, J., and McGlynn, P. (2009). Replication fork reversal and the maintenance of genome stability. Nucleic Acids Res. 37, 3475–3492.

Aze, A., Zhou, J.C., Costa, A., and Costanzo, V. (2013). DNA replication and homologous recombination factors: acting together to maintain genome stability. Chromosoma 122, 401–413.

Becker, J.R., Nguyen, H.D., Wang, X., and Bielinsky, A.K. (2014). Mcm10 deficiency causes defective-template switching and a dependency on error-free postreplicative repair. Cell Cycle 13, 1737–1748.

Bermejo, R., Capra, T., Jossen, R., Colosio, A., Frattini, C., Carotenuto, W., Cocito, A., Doksani, Y., Klein, H., Gómez-González, B., et al. (2011). The replication checkpoint protects fork stability by releasing transcribed genes from nuclear pores. Cell 146, 233–246.

Branzei, D. (2011). Ubiquitin family modifications and template switching. FEBS Lett. 585, 2810–2817.

Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. Nat. Rev. Mol. Cell Biol. 11, 208–219.

Branzei, D., Vanoli, F., and Foiani, M. (2008). SUMOylation regulates Rad18-mediated template switch. Nature 456, 915–920.

Cejka, P., Vondrej, V., and Storchova, Z. (2001). Dissection of the functions of the Saccharomyces cerevisiae Rad6 postreplicative repair group in mutagenesis and UV sensitivity. Genetics 159, 953–963.

Costantino, L., Sotriou, S.K., Rantala, J.K., Magin, S., Mladenov, E., Helleday, T., Haber, J.E., Ilakis, G., Kallioniemi, O.P., and Halazonetis, T.D. (2014). Break-induced replication repair of damaged forks induces genomic duplications in human cells. Science 343, 88–91.

Cotta-Ramusino, C., Fachiineti, D., Lucca, C., Doksani, Y., Lopes, M., Sogo, J., and Foiani, M. (2005). Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mol. Cell 17, 153–159.

Covo, S., Westmoreland, J.W., Gordenin, D.A., and Resnick, M.A. (2010). Cohesin is limiting for the suppression of DNA damage-induced recombination between homologous chromosomes. PLoS Genet. 6, e1001006.

Doksani, Y., Bermejo, R., Fiorani, S., Haber, J.E., and Foiani, M. (2009). Replicon dynamics, dormant origin firing, and terminal fork integrity after double-strand break formation. Cell 137, 247–258.

Gambus, A., van Deursen, F., Polychronopoulos, D., Foltman, M., Jones, R.C., Edmondson, R.D., Calzada, A., and Labib, K. (2009). A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome. EMBO J. 28, 2992–3004.

Gerbi, S.A., and Bielinsky, A.K. (2002). DNA replication and chromatin. Curr. Opin. Genet. Dev. 12, 234–248.

Giannattasio, M., Zwicky, K., Follonier, C., Foiani, M., Lopes, M., and Branzei, D. (2014). Visualization of recombination-mediated damage bypass by template switching. Nat. Struct. Mol. Biol. 21, 884–892.

Gonzalez-Huici, V., Szakal, B., Uralugodi, M., Psakhye, I., Castellucci, F., Menolfi, D., Rajakumarra, E., Fumasoni, M., Bermejo, R., Jentsch, S., and Branzei, D. (2014). DNA bending facilitates the error-free DNA damage tolerance pathway and upholds genome integrity. EMBO J. 33, 327–340.

Halazonetis, T.D., Gorgoulis, V.G., and Bartek, J. (2008). An oncogene-driven DNA damage model for cancer development. Science 319, 1352–1355.

Hanna, J.S., Kroll, E.S., Lundblad, V., and Spencer, F.A. (2001). Saccharomyces cerevisiae CTF18 and CTF4 are required for sister chromatid cohesion. Mol. Cell. Biol. 21, 3144–3158.

Heller, R.C., and Marians, K.J. (2006). Replisome assembly and the direct restart of stalled replication forks. Nat. Rev. Mol. Cell Biol. 7, 932–943.

Hoeger, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 419, 135–141.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071–1078.

Karras, G.I., Fumasoni, M., Sienzki, G., Vanoli, F., Branzei, D., and Jentsch, S. (2013). Noncanonical role of the 9-1-1 clamp in the error-free DNA damage tolerance pathway. Mol. Cell. Biol. 49, 536–546.

Kerzendorfer, C., Cognagli, R., Abramowicz, I., Carpenter, G., and O’Driscoll, M. (2013). Meier-Görlin syndrome and Wolf-Hirschhorn syndrome: two developmental disorders highlighting the importance of efficient DNA replication for normal development and neurogenesis. DNA Repair (Amst.) 12, 637–644.

Kopprina, R.R., Krol, R., Bannikov, V., Bliskovskiy, V., Giziatullin, R., Kirillov, A., Shetopanov, B., Zakhariev, V., Hieter, P., Spencer, F., et al. (1992). CTF4 (CHL15) mutants exhibit defective DNA metabolism in the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. 12, 5736–5747.

Krog, B.O., and Szymington, L.S. (2004). Recombination proteins in yeast. Annu. Rev. Genet. 38, 233–271.

Lehmann, A.R., and Fuchs, R.P. (2006). Gaps and forks in DNA replication: Rediscovering old models. DNA Repair (Amst.) 5, 1495–1498.

Liberi, G., Maffioletti, G., Lucca, C., Chioio, I., Baryshnikova, A., Cotta-Ramusino, C., Lopes, M., Peliolci, A., Haber, J.E., and Foiani, M. (2005). Rad51-dependent DNA structures accumulate at damaged replication forks in sgs1 mutants defective in the yeast ortholog of BLM RecQ helicase. Genes Dev. 19, 339–350.

Liu, X., Wang, X., Reyes-Lamothe, R., and Sherratt, D. (2010). Replication-directed sister chromosome alignment in Escherichia coli. Mol. Microbiol. 75, 1090–1097.

Longhese, M.P., Jovine, L., Plevani, P., and Luccinilli, G. (1993). Conditional mutations in the yeast DNA primase genes affect different aspects of DNA metabolism and interactions in the DNA polymerase alpha-primase complex. Genetics 133, 183–191.
Lopes, M., Foiani, M., and Sogo, J.M. (2006). Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. Mol. Cell 21, 15–27.

Lucchini, G., Mazza, C., Scacheri, E., and Plevani, P. (1988). Genetic mapping of the Saccharomyces cerevisiae DNA polymerase I gene and characterization of a pol1 temperature-sensitive mutant altered in DNA primase-polymerase complex stability. Mol. Gen. Genet. 212, 459–465.

Lydeard, J.R., Lipkin-Moore, Z., Sheu, Y.J., Stillman, B., Burgers, P.M., and Haber, J.E. (2010). Break-induced replication requires all essential DNA repair factors except those specific for pre-RC assembly. Genes Dev. 24, 1133–1144.

Marini, F., Pelliccioni, A., Piaciotti, V., Lucchini, G., Plevani, P., Stern, D.F., and Foiani, M. (1997). A role for DNA primase in coupling DNA replication to DNA damage response. EMBO J. 16, 639–650.

Mclellan, J.L., O’Neill, N.J., Barrett, I., Ferree, E., van Pel, D.M., Ushey, K., Sipahimalani, P., Bryant, J., Rose, A.M., and Hieter, P. (2012). Synthetic lethality of cohesins with PARPs and replication fork mediators. PLoS Genet. 8, e1002574.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell 91, 35–45.

Neelsen, K., and Lopes, M. (2015). Replication fork reversal in euchromatids: from dead end to dynamic response. Nat. Rev. Mol. Cell Biol. http://dx.doi.org/10.1038/nrm3935.

Neelsen, K.J., Zanini, I.M., Herrador, R., and Lopes, M. (2013). Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates. J. Cell Biol. 200, 699–708.

Neelsen, K.J., Chauhduri, A.R., Follonier, C., Herrador, R., and Lopes, M. (2014). Visualization and interpretation of eukaryotic DNA replication intermediates in vivo by electron microscopy. Methods Mol. Biol. 1094, 177–208.

O’Driscoll, M., and Jeggo, P.A. (2008). The role of the DNA damage response pathways in brain development and microcephaly: insight from human disorders. DNA Repair (Amst.) 7, 1039–1050.

Ogiwara, H., Uli, A., Lai, M.S., Enomoto, T., and Seki, M. (2007). Chl1 and Ctf4 are required for damage-induced recombinations. Biochem. Biophys. Res. Commun. 354, 222–226.

Onoda, F., Seki, M., Miyajima, A., and Enomoto, T. (2000). Elevation of sister chromatid exchange in Saccharomyces cerevisiae sgs1 disruptants and the relevance of the disruptants as a system to evaluate mutations in Bloom’s syn- drome gene. Mutat. Res. 459, 203–209.

Papoulil, E., Chen, S., Davies, A.A., Huttnner, D., Krejci, L., Sung, P., and Ulrich, H.D. (2005). Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. Mol. Cell 19, 123–133.

Pfander, B., Moldovan, G.L., Sacher, M., Hoege, C., and Jentsch, S. (2005). SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. Nature 436, 428–433.

Putnam, C.D., Hayes, T.K., and Kolodner, R.D. (2010). Post-replication repair suppresses duplication-mediated genome instability. PLoS Genet. 6, e1000933.

Ray Chauhduri, A., Hashimoto, Y., Herrador, R., Neelsen, K.J., Fachinetti, D., Bermejo, R., Cocito, A., Costanzo, V., and Lopes, M. (2012). Topoisomerase I poisoning results in PARP-mediated replication fork reversal. Nat. Struct. Mol. Biol. 19, 417–423.

Sale, J.E. (2012). Competition, collaboration and coordination—determining how cells bypass DNA damage. J. Cell Sci. 125, 1633–1643.

Simon, A.C., Zhou, J.C., Perera, R.L., van Deursen, F., Evrin, C., Ivanova, M.E., Kilkmenny, M.L., Renault, L., Kjaer, S., Matak-Vinkovic, D., et al. (2014). A Ctf4 trimer couples the OMG helicase to DNA polymerase α in the eukaryotic repli- some. Nature 510, 293–297.

Sogo, J.M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 297, 599–602.

Song, W., Dominska, M., Greenwell, P.W., and Petes, T.D. (2014). Genome-wide high-resolution mapping of chromosome fragile sites in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 111, E2210–E2218.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996). GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. Curr. Biol. 6, 1589–1606.

Suzuki, M., Niimi, A., Limsiarchakul, S., Tomida, S., Miao Huang, Q., Izuta, S., Usukura, J., Itoh, Y., Hishida, T., Akashi, T., et al. (2009). PCNA mono-ubiquitination and activation of translesion DNA polymerases by DNA polymerase alpha. J. Biochem. 146, 13–21.

Szakal, B., and Branzei, D. (2013). Premature Cdk1/Cdc5/Mus81 pathway activation induces aberrant replication and deleterious crossover. EMBO J. 32, 1155–1167.

Tanaka, H., Katou, Y., Yagura, M., Saitoh, K., Itoh, T., Araki, H., Bando, M., and Shirahige, K. (2009). Ctf4 coordinates the progression of helicase and DNA polymerase alpha. Genes Cells 14, 807–820.

Tittel-Elmer, M., Lengronne, A., Davidson, M.B., Bacal, J., Francois, P., Hohl, M., Petrin, J.H., Pasero, P., and Cobb, J.A. (2012). Cohesin association to replication sites depends on rad50 and promotes fork restart. Mol. Cell 48, 98–108.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister-chromatid separa- tion at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature 400, 37–42.

van der Lelij, P., Oostra, A.B., Rooman, M.A., Joenje, H., and de Winter, J.P. (2010). Diagnostic Overlap between Fanconi Anemia and the Cohesinopathies: Roberts Syndrome and Warsaw Breakage Syndrome. Anemia 2010, 565288.

Vanoli, F., Fumasoni, M., Szakal, B., Maloisel, L., and Branzei, D. (2010). Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch. PLoS Genet. 6, e1001255.

Warren, C.D., Eckley, D.M., Lee, M.S., Hanna, J.S., Hughes, A., Peyser, B., Jie, C., Irizarry, R., and Spencer, F.A. (2004). S-phase checkpoint genes safeguard high-fidelity sister chromatid cohesion. Mol. Biol. Cell 15, 1724–1735.

Waters, L.S., and Walker, G.C. (2006). The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G2/M phase rather than S phase. Proc. Natl. Acad. Sci. USA 103, 8971–8976.

Xu, H., Boone, C., and Brown, G.W. (2007). Genetic dissection of parallel sister-chromatid cohesion pathways. Genetics 176, 1417–1429.

Yoshizawa-Sugata, N., and Masai, H. (2009). Roles of human AND-1 in chromo- some transactions in S phase. J. Biol. Chem. 284, 20718–20728.
Supplemental Information

Error-Free DNA Damage Tolerance and Sister Chromatid Proximity during DNA Replication

Rely on the Polα/Primase/Ctf4 Complex

Marco Fumasoni, Katharina Zwicky, Fabio Vanoli, Massimo Lopes, and Dana Branzei
SUPPLEMENTARY INFORMATION

Contains 5 figures, 1 table, methods, and references.

Figure S1. Related to Figure 1.

Figure S1. Genetic analysis of the DNA damage tolerance defects of ctf4Δ cells. (A) WT (FY1000), ctf4Δ (HY2193), sgs1 (HY1461) and sgs1 ctf4Δ
(HY1472) cells were synchronized in G2/M at 25°C prior to release at 30°C in MMS-containing media (0.033%), samples for FACS analysis were taken every 15 minutes. (B) WT (FY1000) and ctf4Δ (HY2193) were synchronized in G2 at 25°C prior to release at 30°C in MMS-containing media (0.033%). The genomic DNA was psoralen crosslinked in vivo before extraction, digested with EcoRV/HindIII and analyzed by 2D gel with a probe against ARS305. Plots with the cell-cycle profiles and quantifications of the replication intermediates are displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges. Spot assay analysis of (C) WT (FY0001), rev3Δ (HY0008), ctf4Δ (HY3466), ctf4Δ rev3Δ (HY3468), (D) WT (FY1000), ctf4Δ (HY2193), rad51Δ (FY0115), rad18Δ (HY0139), ctf4Δ rad51Δ (HY1805), ctf4Δ rad18Δ (HY2094), rad51Δ rad18Δ (HY2794), ctf4Δ rad51Δ rad18Δ (HY2788), (E) WT (FY1000), ctf4Δ (HY2193), rad18Δ (HY0139), mms2Δ (HY0922), rad5Δ (HY0137), ctf4Δ rad18Δ (HY2094), ctf4Δ mms2Δ (HY2169), ctf4Δ rad5Δ (HY2229). Serial dilutions of cells were spotted on plates containing MMS at the indicated concentrations and incubated at 30°C for three days.
Figure S2. Polα/Primase mutants have template switch and cohesion defects. (A) *sgs1* (HY1461), *sgs1 pol1-1* (HY1458) cells were synchronized in G2 prior to release at 30°C in MMS containing medium (0.033% MMS). The genomic DNA extracted from samples collected at the indicated time points was digested with *NcoI* and analyzed by 2D gel with a probe against *ARS305*. Plots with the cell-cycle profiles and quantifications of the replication intermediates are displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges. (B) Tetrad dissection of *pri1-M4 X ctf18Δ* and *pri1-M4 X ctf8Δ* crossings, expected double mutants are indicated by the orange circles. The line indicates elimination of superfluous lanes from the tetrad dissection plate.
Figure S3. Related to Figure 3.

Figure S3. Specificity validation of the LacO/LacI tethering system. The DNA of the experiment showed in Figure 3 was re-digested and analyzed with probes specific for the LEU2 (in proximity to the LacO repeats) or ARS1 locus, on chromosome IV. Quantifications of the replication intermediates are displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges.
Figure S4. Related to Figure 4.

**Figure S4. Cohesin and Polα/Primase/Ctf4 functions in recombination-mediated DNA damage tolerance.** (A) 2D gel analysis of replication intermediates from sgs1Δ and sgs1Δ rad51Δ carrying the LacO repeats and the dimeric (HY1861, HY2083) or the tetrameric (HY1859, HY2084) LacI version. The cells were grown and arrested in G1 at 25°C. Cells were transferred to synthetic complete media lacking histidine and containing 10 mM 3-aminotriazole and α-factor for the last 20 min of the arrest in order to induce the expression of LacI. Cells were then released in medium containing 0.033% MMS at 30°C. 2D gel analysis was conducted as in the Figure 4.

Plots with the cell-cycle profiles and quantifications of the X molecules are
displayed. The columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges. (B) Tetrad dissection of ctf4Δ × scc1-73 crossing, with expected genotypes. (C) Damage sensitivity measured by spot assay of WT (FY1000), pri1-M4 (HY1607), scc1-73 (HY1932) and pri1-M4 scc1-73 (HY4051): serial dilutions were plated on YPD or MMS containing plates at the indicated concentrations and incubated at 30° for three days.
Figure S5. Related to Figure 5.

Figure S5. Recombination pathways providing for viability in pri1-M4 mutants. Tetrad dissection of (A) pri1-M4 X rad52Δ and pri1-M4 X rad51Δ. The line indicates elimination of superfluous lanes from the tetrad dissection plate image. (B) ctf4Δ X rad1Δ crossings. The expected genotype is indicated close to the images.
Table S1. Related to Figures 1-6. List of *Saccharomyces cerevisiae* yeast strains used in this study.

| NUMBER | GENOTYPE | ORIGIN |
|--------|----------|--------|
| FY1000 (W303) | MATα ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, can1-100, RAD5+ | Lab collection |
| FY0001 (PY83) | MATα ade2-101 leu2-3,112 lys2-801 ura3-52 his3-Δ200, RAD5+ | Lab collection |
| HY1788 | MATα, ade2-1, trp1-1, leu2-3,112, ura3, can1-100, his3-11,15::HIS3tetR-GFP, ura3::3XURA3tetO112, omns | K. Nasmyth |
| FY1362 | MATα, can1-100, his3-11-15, leu2-3-112, trp1-1, can1-1, GFP-LACI::HIS3 (pAFS78), LacO::LEU2(pAFS59) | A.W. Murray |
| FY1361 | MATα, can1-100, his3-11-15, leu2-3-112, trp1-1, can1-1, GFP-LACI::HIS3 (pAFS87), LacO::LEU2(pAFS59) | A.W. Murray |
| HY1461 | W303, MATα, sgs1Δ::AUR1 | This study |
| HY1472 | W303, MATα sgs1Δ::AUR1, ctf4Δ::TRP | This study |
| HY0008 | PY83, MATα, rev3Δ::LEU2 | Lab collection |
| HY3466 | PY83, MATα, ctf4Δ::hphMX4 | This study |
| HY3468 | PY83 MATα, rev3Δ::LEU2, ctf4Δ::HPHMX4 | This study |
| HY2193 | W303, MATα, ctf4Δ::TRP1 | This study |
| FY1060 | W303, MATα, sgs1Δ::HIS3MX6 | Lab collection |
| HY1934 | W303, MATα, scc1-73, sgs1Δ::HIS3MX6 | This study |
| HY1457 | W303, MATα, pri1::pri1-M4-URA3, sgs1Δ::AUR1 | This study |
| HY1853 | HY1788, MATα, ctf4Δ::TRP1 | This study |
| HY1872 | HY1788, MATα, pri1::pri1-M4-URA3 | This study |
| HY1823 | HY1788, MATα, chl1Δ::TRP1 | This study |
| HY1825 | HY1788, MATα, ctf18Δ::TRP1 | This study |
| HY4252 | FY1362, MATα, sgs1Δ::KANMX4 | This study |
| HY4255 | FY1362, MATα, sgs1Δ::KANMX4, scc1-73 | This study |
| HY4259 | FY1361, MATα, sgs1Δ::KANMX4 | This study |
| HY4262 | FY1361, MATa, sgs1::KANMX4, scc1-73 | This study |
| HY3979 | FY1362, MATa, sgs1::AUR1 | This study |
| HY3981 | FY1362, MATa, sgs1::AUR1, ctf4::TRP | This study |
| HY3983 | FY1361, MATa, sgs1::AUR1 | This study |
| HY3985 | FY1361, MATa, sgs1::AUR1, ctf4::TRP | This study |
| HY1607 | W303, MATa, pri1::pri1M4-URA | M. Foiani |
| FY1162 | W303, MATα, leu2-k::URA3-ADE2::leu2-k ura3 | H.Klein |
| FY0115 | W303, MATa, rad51::LEU2 | H.Klein |
| HY0139 | W303, MATa, rad18Δ::HPHX4 | Lab collection |
| HY1805 | W303, MATα, ctf4::TRP1 rad51Δ::LEU2 | This study |
| HY2094 | W303, MATα, rad18Δ::LEU2 ctf4::TRP1 | This study |
| HY2794 | W303, MATα, rad51Δ::LEU2 rad18Δ::HPHX4 | This study |
| HY2788 | W303, MATα, ctf4::TRP1, rad51Δ::LEU2, rad18Δ::HPHX4 | This study |
| HY0922 | W303, MATα, mms2Δ::HPHX6 | Lab collection |
| HY0137 | W303, MATα, rad5Δ::HPHX4 | Lab collection |
| HY2169 | W303, MATα, ctf4Δ::TRP1, mms2Δ::HPHX4 | This study |
| HY2229 | W303, MATα, ctf4Δ::TRP1 rad5Δ::HPHX4 | This study |
| HY1458 | W303, MATα, sgs1Δ::AUR1, pol1-1 | This study |
| HY1932 | W303, MATα, scc1-73 | This study |
| HY4051 | W303, MATα, scc1-73, pri1::pri1M4-URA | This study |
| HY1861 | FY1362, MATα, sgs1Δ::KANMX4 | This study |
| HY2083 | FY1362, MATα, sgs1Δ::KANMX4, rad51Δ::HPHX4 | This study |
| HY1859 | FY1361, MATα, sgs1Δ::KANMX4 | This study |
| HY2084 | FY1361 MATα, sgs1Δ::KANMX4, rad51Δ::HPHX4 | This study |
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Strain construction

*S. cerevisiae* strains were prepared by genetic crosses and standard gene disruption techniques (Goldstein and McCusker, 1999; Wach et al., 1994). Original strains used in the cohesion assays were obtained from (Michaelis et al., 1997) as described, by triple integration of a plasmid carrying 112 tandem repeats of the TetO sequence (p306tetO112) at the URA3 locus. An additional plasmid carrying the TetR fused at the C-terminus with a GFP molecule followed by a nuclear localization signal (NLS) was then introduced at the LEU2 locus. Original strains used in the artificial tethering of the sister chromatids were obtained in a similar manner in (Straight et al., 1996). A plasmid containing 256 tandem repeats of the LacO sequence (pAFS59) was linearized with EcoRV and integrated within the LEU2 locus. A plasmid containing the wild-type form (pAFS67) or the dimeric form (pAFS78) of the LacI, fused with a GFP-NLS sequence, was then integrated at the HIS3 locus by NheI digestion. Strains used in the experiments in Figure 2C (TetO/TetR cohesion assay) and Figures 3, 4, S3 and S4A (LacO/LacI artificial tethering) were obtained by crossings with the relative original strains following the relevant markers. Stability of the tandem repeats after crossing was then checked by Southern blot analysis.
Growing and arrest conditions

Unless otherwise indicated, strains were grown at 25°C in YP media containing 2% of glucose (YPD) as carbon source. Cells were synchronized either in metaphase by adding nocodazole (8 µg/ml) and DMSO (final concentration of 1% v/v), or in G1 with α-factor to a final concentration of 3 µg/ml, with the exception of experiments presented in Figures 3B, 4A, S3 and S4A in which cells were additionally resuspended in synthetic complete media lacking histidine and containing 10 mM 3-aminotriazole during the last 20 min of the arrest to induce LacI expression as previously described (Straight et al., 1996). The release from synchronization was performed as previously described, in YPD containing MMS at a final concentration of 0.033% v/v (Branzei et al., 2006) at temperatures indicated in the figure legends.

Fluorescence-activated cell sorter (FACS) analysis

Cell cycle analysis was conducted as previously described (Vanoli et al., 2010). In brief, 1x10^7 cells were collected by centrifugation, and resuspended in 70% ethanol for 16 h. Cells were then washed in 0.25 M Tris-HCl (pH 7.5), resuspended in the same buffer containing 2 mg/ml of RNaseA and incubated at 37°C for at least 1h. In case of Sytox-green staining, cells were further treated overnight with proteinase K (1 mg/ml) at 37°C. Cells were then resuspended in Tris-HCl (pH 7.5) 200 mM, NaCl 200 mM, MgCl2 80 mM and stained in the same buffer containing 50 µg/ml of propidium-iodide (Sigma) or 1 µM Sytox-green (Invitrogen). Samples were then diluted 10-fold in 50 mM Tris-HCl (pH 7.8) and analyzed using a Becton Dickinson FACScan.
**Drug sensitivity assay**

Cells were grown in YPD at 28°C, if not differently specified, counted and adjusted to a 1x10^8 cells per ml concentration. Serial 1:10 dilutions were made in YPD and one drop of each dilution was pin-spotted onto YPD or YPD containing different MMS concentrations. The plates were then incubated for 2-4 days at 30°C if not otherwise specified.

**Mutagenesis assay**

Spontaneous mutagenesis at the CAN1 locus was assessed by measuring the canavanine resistant fraction of parallel saturated populations as previously described in (Gonzalez-Huici et al., 2014). 10 individual YPD cultures were setup with a 1:20000 dilution from an overnight culture and incubated with constant shaking at 30°C for 36 h in order to induce the acquisition of spontaneous mutations. After appropriate dilutions were made, cells were pelleted, washed with sterile water and plated on YPD plates (200-500 cells) or synthetic complete (SC) media plates lacking Arginine, but containing canavanine (80 μg/ml) (SC-Arg+Can) (10^7- 5X10^7 cells). After 3-4 days of incubation on plates, colonies were counted. At least three independent experiments were performed for each strain. The obtained values were used to estimate spontaneous mutation rates using the maximum-likelihood approach and the FALCOR software (http://www.keshavsingh.org/protocols/FALCOR.html).
**Deletion assay**

The deletion rate assay was performed as previously described in (Aguilera and Klein, 1989; Gomez-Gonzalez et al., 2011). Heterozygous diploid strains were sporulated on VB plates, dissected on YPD plates and grown for 3-4 days at 25°C. Seven single colonies for each strain (carrying the appropriate genotype) were then resuspended in 1 ml of sterile distilled H2O and 4-5 serial dilutions (1:10) were made. Cells were then plated on low-adenine plates containing 9 µg Adenine/ml (~10^3 cells) or on media containing 5-FOA at 1 mg/ml (~10^7 cells), to visualize ADE2^- and URA3^- colonies respectively. Deletion events were scored and multiplied by the appropriate dilution factor to calculate the frequency of recombination. At least three independent experiments were performed for each strain. Values were then utilized for the fluctuation test using the maximum-likelihood approach and the FALCOR software (http://www.keshavsingh.org/protocols/FALCOR.html).

**Protein based procedures**

Proteins were analyzed from denatured yeast crude extracts as previously described (Liberi et al., 2000). Briefly, 10^8 cells/ml were harvested, resuspended in 2 ml of TCA 20% and transferred to 2 ml eppendorf tubes. The pellet was resuspended in 200 µL of TCA 20% and an equal volume of acid-washed glass beads (425-600 µm, Sigma) was added. Cells were broken by continuous vortexing for 2-4 min. 400 µL of TCA 5% was added to have a final concentration of TCA 10%. The lysates were then transferred to
new 1.5 ml tubes and centrifuged for 10 min at 3000 rpm, RT. The pellet was resuspended in 100 μL Laemmli Buffer 1X. The pH was then adjusted with 50μl of Tris Base 1M. The protein extracts were boiled for 3 min and centrifuged for 10 min at 3000 rpm, RT. The supernatant was collected and analyzed by SDS-PAGE. PCNA was detected using the mouse monoclonal antibody from Abcam (ab70472).

**Premature sister chromatid separation assay**

Logarithmically growing cells were treated with 3 μg/ml α-Factor to induce G1 arrest. Cells were then washed using YP and released in YPD containing 10 μg/ml of nocodazole (0.1% DMSO total) in order to allow one round of replication. After three hours of nocodazole treatment, G2 arrest was checked by cell morphology and 20 ml of cells were collected and fixated in 4% formaldehyde for 5min at room temperature. Cells were then collected by centrifugation, resuspended in SK buffer (1M sorbitol, 0.05M K₂PO₄) and sonicated for 8 seconds prior to microscope analysis. Cells were imaged on a Delta-Vision microscope (Applied Precision) using 100X oil immersion lens. Fluorescence was visualized with a conventional FITC excitation filter and a long pass emission filter. Images were analyzed using ImageJ software. Statistical analysis was performed on results obtained in at least three independent experiments.
2D gel and electron microscopy samples preparation

In all, 200 ml cultures (2-4 x 10^9 cells) were arrested by addition of 0.1% sodium azide (final concentration) and cooled down on ice. In case of psoralen crosslinking experiments (indicated in the figure legends), cells were additionally treated using a modified protocol with respect to the one described in (Gasser et al., 1996). Briefly, cells were washed, resuspended in 5ml of cold water in small petri dishes and kept on ice. 300 µl of 4,5',8-tri-methyl-psoralen solution (0.2 mg/ml in EtOH 100%) was added prior to extensive resuspension by pipetting, followed by 5 min of incubation in the dark and then 10 min of UV irradiation at 365 nm (Stratagene UV Stratalinker). The procedure was then repeated 3 times (for 2D gels) or 5 times (for EM analysis) to ensure extensive crosslinking. Cells were then harvested by centrifugation, washed in cold water, and incubated in spheroplasting buffer (1M sorbitol, 100 mM EDTA (pH 8.0), 0.1% β-mercaptoethanol, and 50U zymolyase/ml) for 1.5 h at 30°C. In all, 2ml water, 200µl RNase A (10 µg/ml), and 2.5 ml Solution I (2% w/v cetyl-trimethyl-ammonium-bromide (CTAB), 1.4M NaCl, 100 mM Tris–HCl (pH 7.6), and 25 mM EDTA (pH 8.0)) were sequentially added to the spheroplast pellets and samples were incubated for 30 min at 50°C. 200 ml Proteinase K (20 mg/ml) was then added and the incubation was prolonged at 50°C for 90 min, and at 30°C overnight. The sample was then centrifuged at 4000 r.p.m. for 10 min: the cellular debris pellet was kept for further extraction, while the supernatant was extracted with 2.5 ml chloroform/isoamylalcohol (24:1) and the DNA in the upper phase was precipitated by addition of 2 volumes Solution II (1% w/v
CTAB, 50 mM Tris–HCl (pH 7.6), and 10 mM EDTA) and centrifugation at 8500 r.p.m. for 10 min. The pellet was resuspended in 2 ml Solution III (1.4 M NaCl, 10 mM Tris–HCl (pH 7.6), and 1 mM EDTA). Residual DNA in the cellular debris pellet was also extracted by resuspension in 2 ml Solution III and incubation at 50°C for 30 min, followed by extraction in 1 ml chloroform/isoamylalcohol (24:1). The upper phase was pooled together with the main DNA prep. Total DNA was then precipitated with 1 volume of isopropanol, washed with 70% ethanol, air-dried, and finally resuspended in TE 1X. Signals were detected following 2D gel electrophoresis and standard southern blot procedures using probes against ARS305 (Chr III 39002-40063), LEU2 flanking region (Chr III 95697-97161) and ARS1 (Chr IV 461864-463123).

Quantification of replication intermediates
Quantification of X-shaped intermediate signals was performed using the Image Quant software (GE Healthcare) as previously described (Vanoli et al., 2010). For each time point, areas corresponding to the monomer spot (M), the X-signal and a region without any replication intermediates as background reference were selected and the signal intensities (SI) in percentage of each signal were obtained (see example below for selection of monomer spot, spike, background or total signals). The values for the X and monomer were then corrected by subtracting from the SI value the background value after the latter was multiplied for the ratio between the dimension of the area for the intermediate of interest and for
background (see example of working sheet below). The relative signal intensity for the X was determined by dividing the value for X with the sum of the X and monomer values. The resulting values for X signals were then normalized and converted to percentage by using the highest value number of X for each experiment as 100 and normalizing the other values to it.
We found very similar final values when the X-signal was normalized to the total signal (T) instead of the monomer spot as shown in the example we provided. As sometimes in the total area unrelated signals appear (likely due to technical issues related to hybridization or washes of the filters), the values reported in this study used the normalization versus the monomer spot.

We also note that slight differences in various steps required for a 2D analysis (different genomic locations that also impose different running conditions, different temperatures or synchronization procedure) may generate variability in the absolute numbers obtained with our quantification tools. The differences reported in our analysis are however clearly detectable and highly reproducible (in independent experiments, at various time points) as shown by the normalized quantification and by the ranges displayed on the quantification graphs.

**Electron microscopy analysis**

Genomic DNA was extracted using the CTAB-Psoralen procedure and then enriched for replication intermediates (RIs) as described previously (Neelsen et al., 2014). Briefly, 15 μg of DNA for each strain were digested with *Pvu*I for 3 h following manufacturer’s instructions and additionally treated with RNAsesIII to avoid RNA contamination of the samples. The digestion mix, adjusted to 300 mM NaCl was then loaded on a chromatography column containing 1 ml of BND cellulose stock (0.1 g/column; Sigma B-6385, pre-equilibrated with 10 mM Tris-HCl pH 8, 300 mM NaCl). DNA was incubated with the BND cellulose for 30 min with resuspension every 10 min to allow full
binding of the DNA molecules and flow-through was collected by gravity flow. Twice 1 ml of 10 mM Tris-HCl pH 8 containing 1M NaCl was added to the column to collect linear double-stranded molecules (salt elution, 70-90% of total DNA). 600 ml of 10 mM Tris-HCl pH 8, 1M NaCl containing 1.8% caffeine were finally added, incubated for 10 min, in order to induce elution of the RIs. DNA was then purified and concentrated using conical Amicon Ultra centrifugal filters (0.5 ml 100K-MWCO 100K) following manufacturer’s instructions. Fractions of the samples were then spread onto carbon-coated EM grids in the presence of uranyl acetate followed by platinum-based rotatory shadowing and analyzed as described in (Neelsen et al., 2014).

The assignment criteria for single-stranded regions on the DNA molecules analyzed in this work were recently described (Neelsen et al., 2014). We note that in order to assign a ssDNA region on a DNA filament it is necessary to identify two points on the DNA molecule that define the borders of the ssDNA region, in which the thickness of the DNA filament (in our experimental conditions ~20 Angstroms) decreases close to one half. We note that the observed thickness of the molecules is largely determined by the amount of deposited heavy atoms during the shadowing procedure. The length measurements were performed using a conversion factor expressed in graphic units per base pairs/nucleotides (automatically converted in nanometers/nucleotide according to the magnification value at which the picture was taken), using a plasmid of known length as internal standard (Neelsen et al., 2014).
Supplementary References

Aguilera, A., and Klein, H.L. (1989). Genetic and molecular analysis of recombination events in Saccharomyces cerevisiae occurring in the presence of the hyper-recombination mutation hpr1. Genetics 122, 503-517.

Branzei, D., Sollier, J., Liberi, G., Zhao, X., Maeda, D., Seki, M., Enomoto, T., Ohta, K., and Foiani, M. (2006). Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks. Cell 127, 509-522.

Gasser, R., Koller, T., and Sogo, J.M. (1996). The stability of nucleosomes at the replication fork. J. Mol. Biol. 258, 224-239.

Goldstein, A.L., and McCusker, J.H. (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15, 1541-1553.

Gomez-Gonzalez, B., Ruiz, J.F., and Aguilera, A. (2011). Genetic and molecular analysis of mitotic recombination inSaccharomyces cerevisiae. Methods Mol. Biol. 745, 151-172.

Gonzalez-Huici, V., Szakal, B., Urulangodi, M., Psakhye, I., Castellucci, F., Menolfi, D., Rajakumara, E., Fumasoni, M., Bermejo, R., Jentsch, S., et al. (2014). DNA bending facilitates the error-free DNA damage tolerance pathway and upholds genome integrity. EMBO J. 33, 327-340.

Liberi, G., Chiolo, I., Pellicioli, A., Lopes, M., Plevani, P., Muzzi-Falconi, M., and Foiani, M. (2000). Srs2 DNA helicase is involved in checkpoint response and
its regulation requires a functional Mec1-dependent pathway and Cdk1 activity. EMBO J 19, 5027-5038.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell 91, 35-45.

Neelsen, K.J., Chaudhuri, A.R., Follonier, C., Herrador, R., and Lopes, M. (2014). Visualization and interpretation of eukaryotic DNA replication intermediates in vivo by electron microscopy. Methods Mol. Biol. 1094, 177-208.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996). GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. Curr. Biol. 6, 1599-1608.

Vanoli, F., Fumasoni, M., Szakal, B., Maloisel, L., and Branzei, D. (2010). Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch. PLoS Genetics 6, e1001205.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast 10, 1793-1808.