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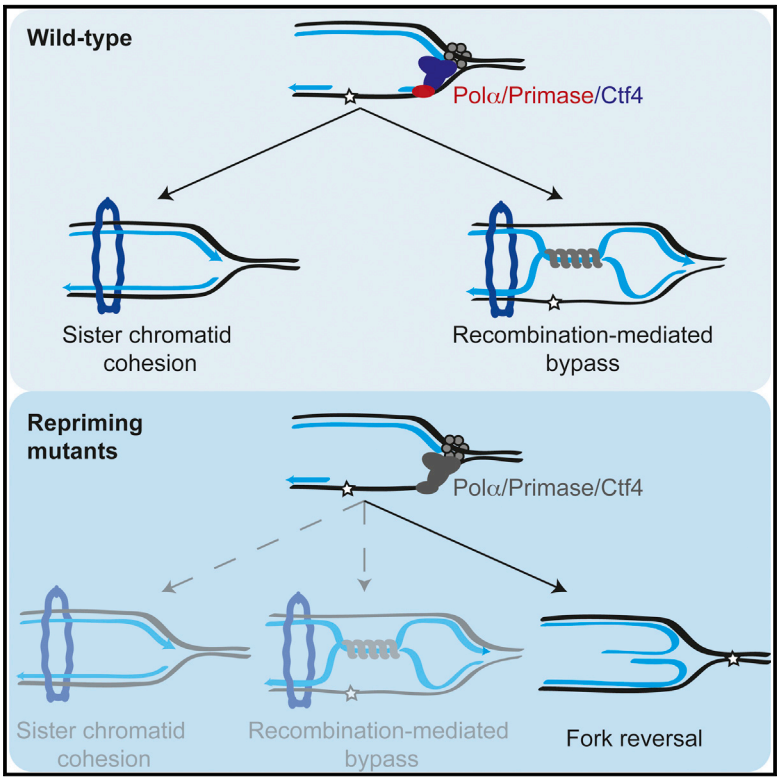
Molecular Cell

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

Graphical Abstract



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

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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.

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

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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 chromatid 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'Driscoll 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 Polα. These RNA-DNA primers are then extended by the replicative polymerases Polε and Polδ (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.

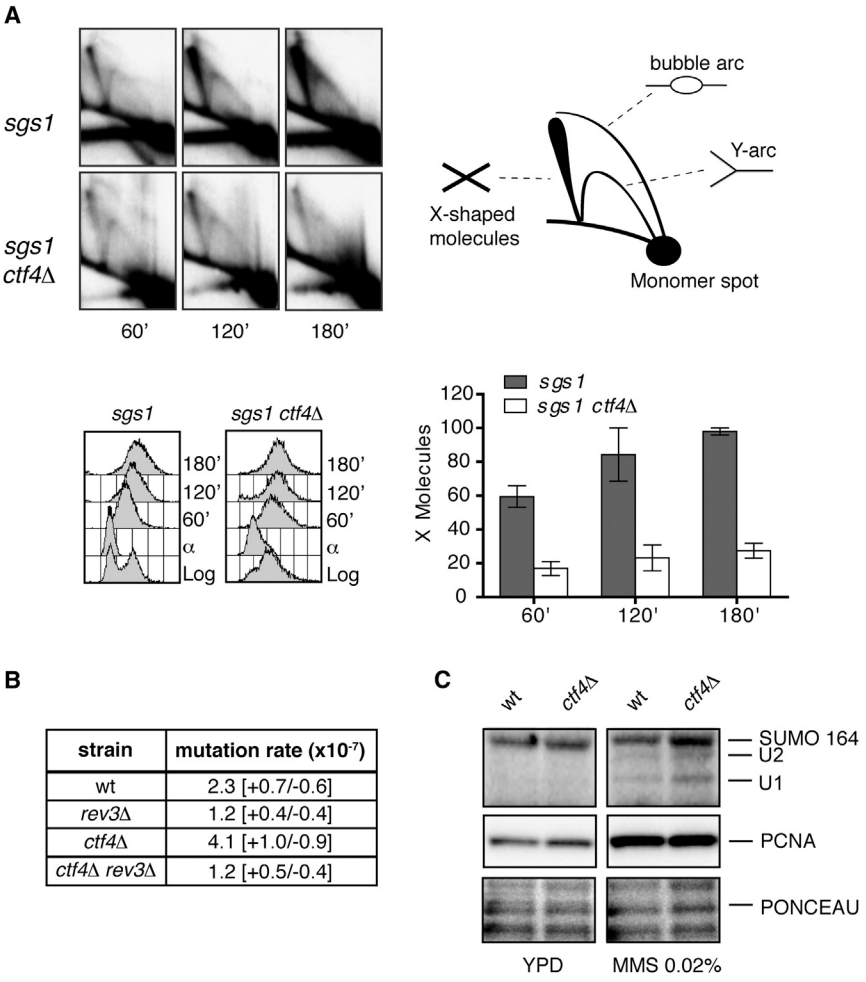
Cells defective in error-free DDT are characterized by a more prominent dependency on TLS, showing higher levels of spontaneous mutagenesis and increased damage sensitivity following inactivation of error-prone DDT components (Cejka et al., 2001). We found that *ctf4Δ* cells have statistically significant 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.



strain	mutation rate (x10^{-7})
wt	2.3 [+0.7/-0.6]
rev3Δ	1.2 [+0.4/-0.4]
ctf4Δ	4.1 [+1.0/-0.9]
ctf4Δ rev3Δ	1.2 [+0.5/-0.4]

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 *Nco*I 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 (×10^{-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.

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 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 complex. To this purpose, 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).

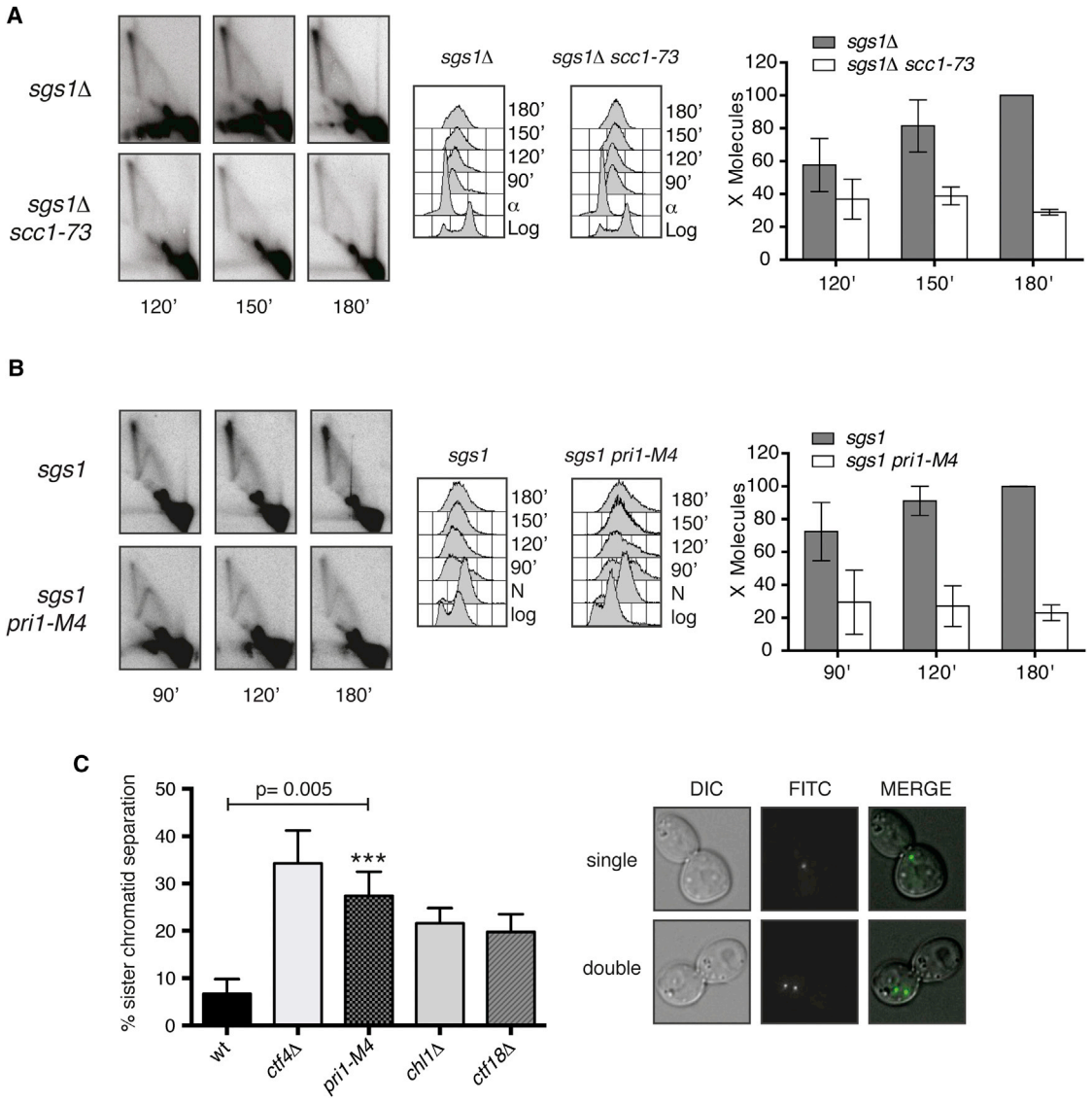


Figure 2. Primase and Cohesin Mutants Are Characterized by Defects in Cohesion and Template Switching

(A) *sgs1Δ* (FY1060) and *sgs1Δ scc1-73* (HY1934) cells were synchronized in G1 with alpha-factor (α) at 25°C and released in media containing 0.033% MMS at 37°C. The genomic DNA was digested with *Eco*RV-*Hind*III and analyzed by 2D gel with a probe against *ARS305*. FACS and X-molecule quantification plots are shown.

(B) *sgs1* (HY1461) and *sgs1 pri1-M4* (HY1457) were synchronized in G2/M with nocodazole (N) prior to release at 28°C in media containing 0.033% MMS. The genomic DNA was digested with *Eco*RV-*Hind*III and analyzed with a probe against *ARS305*. FACS and X-molecule quantification plots are displayed. In both (A) and (B), the columns in the quantification graphs denote the data mean of two independent experiments and the bars indicate ranges.

(C) Cohesion assay: WT (HY1788), *ctf4Δ* (HY1853), *pri1-M4* (HY1872), *chl1Δ* (HY1823), and *ctf18Δ* (HY1825) cells were arrested in G1 at 25°C and released in nocodazole-containing medium for 3 hr at 30°C. The histogram shows the mean and SD of the percentage of cells showing two dots. The p value and asterisks indicating highly significant statistical difference are demonstrated for the *pri1-M4* allele. The cohesion defects of *chl1Δ*, *ctf4Δ*, and *ctf18Δ* strains were also statistically significant, as previously reported (Xu et al., 2007). The differences in the cohesion defects of *pri1-M4* and the *chl1Δ*, *ctf4Δ*, and *ctf18Δ* control strains were not statistically significant. Representation of the cells proficient (one dot; single) and deficient (two dots; double) in sister chromatid cohesion is shown. See also Figure S2.

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

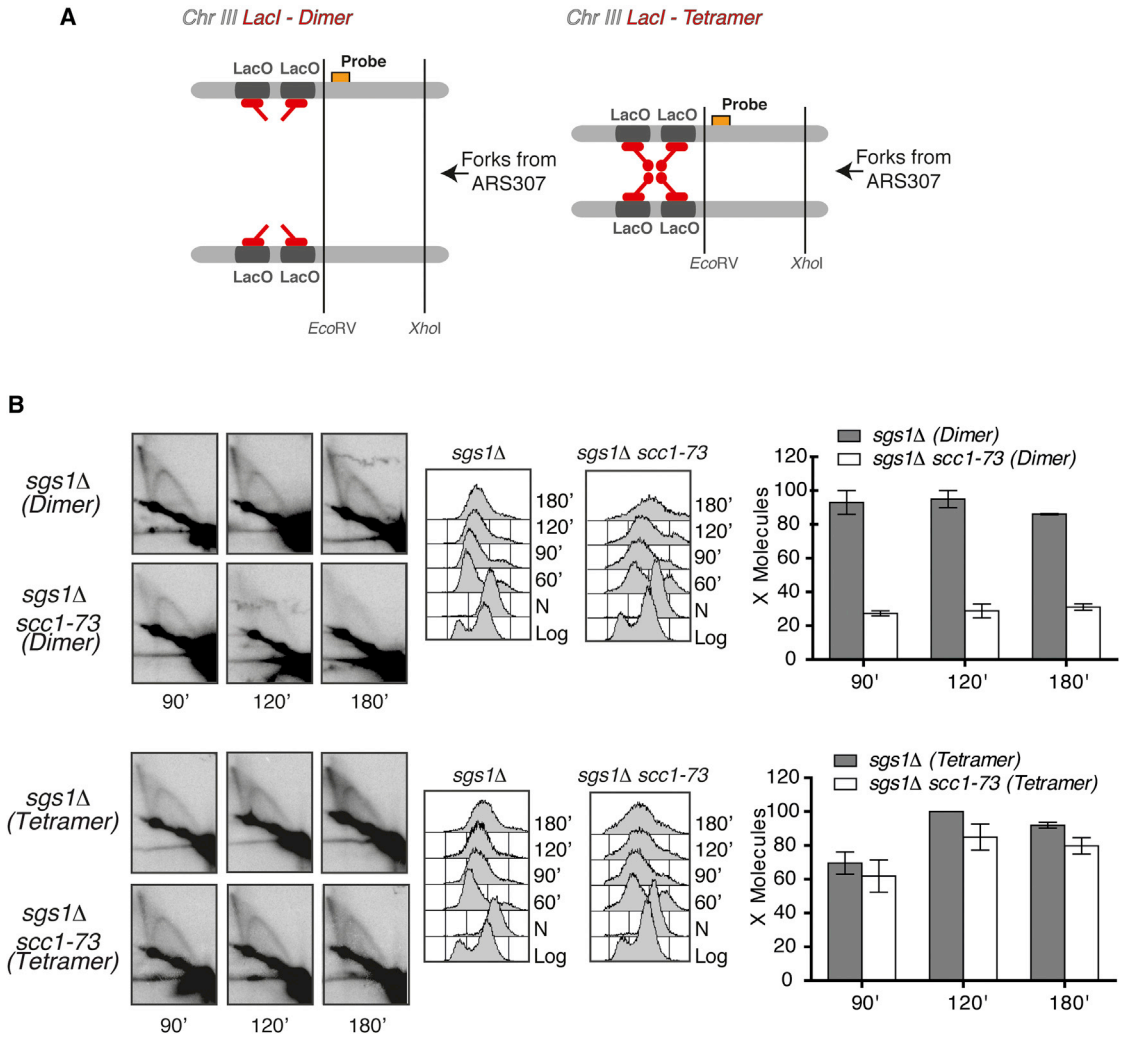


Figure 3. Artificial Tethering of Sister Chromatids Suppresses the Template Switching Defects of Cohesin Mutants

(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 *Eco*RV/*Xho*I 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.

the accessibility of the sister chromatid donor sequence cohesin facilitates sister chromatid recombination (Covo et al., 2010; Tittel-Elmer 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

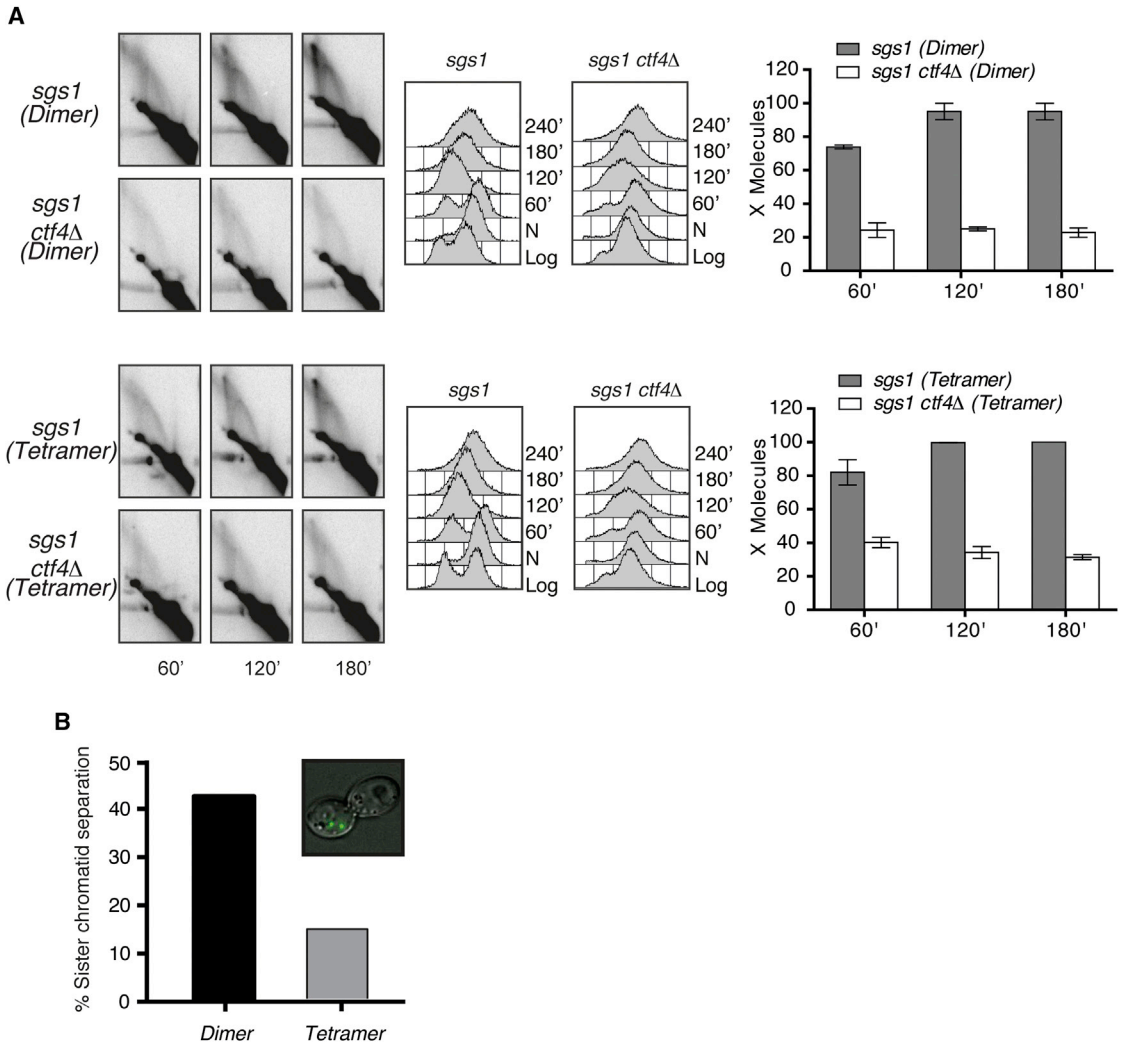


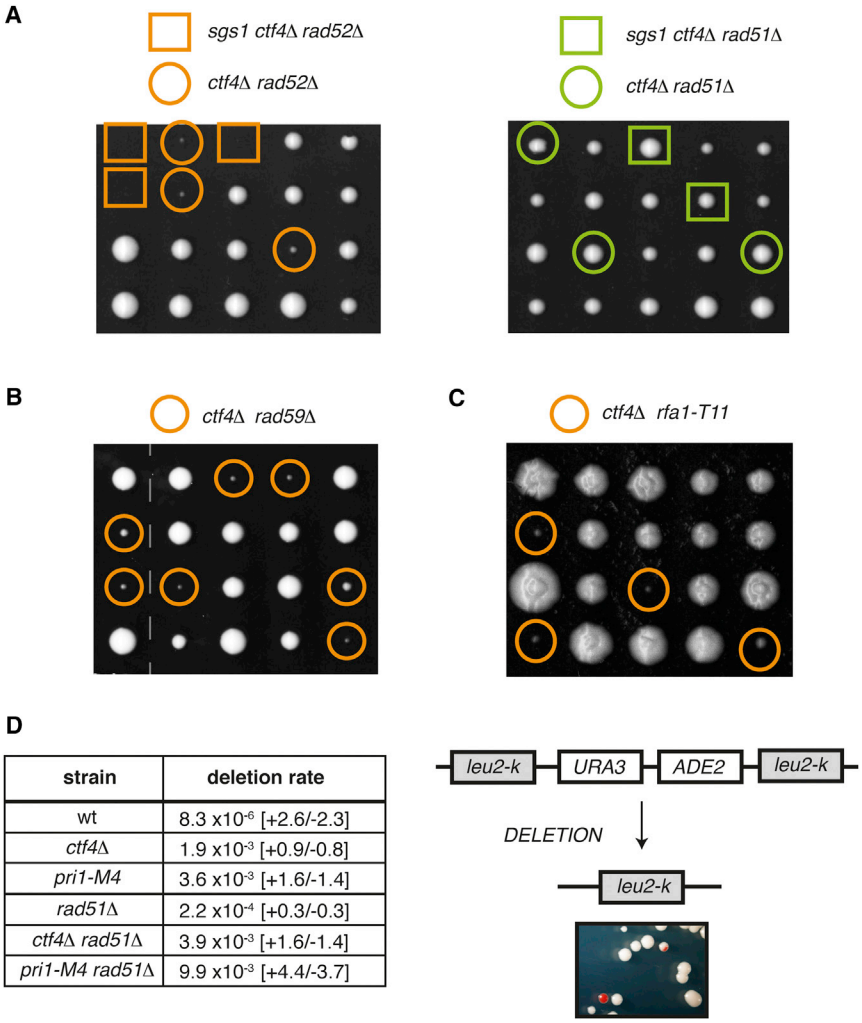
Figure 4. Artificial Cohesion Does Not Restore Efficient Template Switching in *ctf4*Δ Mutants

(A) *sgs1* and *sgs1 ctf4*Δ 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 ctf4Δ* cells carrying the dimeric and tetrameric version of the LacI. The experiment was conducted as in Figure 2C. See also Figure S4.

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 *sgs1Δ 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 *ctf4Δ* defect in TS is also due to increased physical distance between the sister chromatids, we used the same experimental approach, but in the *sgs1 ctf4Δ* background. Interestingly, artificial cohesion did not restore the formation of X molecules in the *sgs1 ctf4Δ* strain (Figure 4A), although the LacO/LacI system efficiently reduced the local cohesion defects on chromosome III associated with *ctf4Δ* (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 *ctf4Δ* cells



strain	deletion rate
wt	8.3 x10^{-6} [+2.6/-2.3]
ctf4Δ	1.9 x10^{-3} [+0.9/-0.8]
pri1-M4	3.6 x10^{-3} [+1.6/-1.4]
rad51Δ	2.2 x10^{-4} [+0.3/-0.3]
ctf4Δ rad51Δ	3.9 x10^{-3} [+1.6/-1.4]
pri1-M4 rad51Δ	9.9 x10^{-3} [+4.4/-3.7]

Figure 5. Recombination Pathways Supporting the Viability of *CTF4* Mutants

(A–C) Tetrad dissection of *ctf4Δ X sgs1 rad52Δ* and *ctf4Δ X sgs1 rad51Δ* (A), *ctf4Δ X rad59Δ* (B), and *ctf4Δ X rfa1-T11* (C) crossings. The expected genotypes are indicated. In (B), the line indicates elimination of superfluous lanes from the tetrad dissection plate image.

(D) Deletion rate assay: single colonies of WT, *ctf4Δ*, *pri1-M4*, *rad51Δ*, *ctf4Δ rad51Δ*, and *pri1-M4 rad51Δ* cells (obtained from crossing FY1162 carrying the direct repeat construct, with *ctf4Δ rad51Δ* and *pri1-M4 rad51Δ*, respectively) were suspended in water and diluted before being plated on YPD, 5-FOA and low-adenine plates. Red sectors/colonies characteristic of deletion events are shown. Intra-chromosomal deletion rates and 95% confidence intervals were estimated using the maximum-likelihood method. See also Figure S5.

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* even at permissive temperatures (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

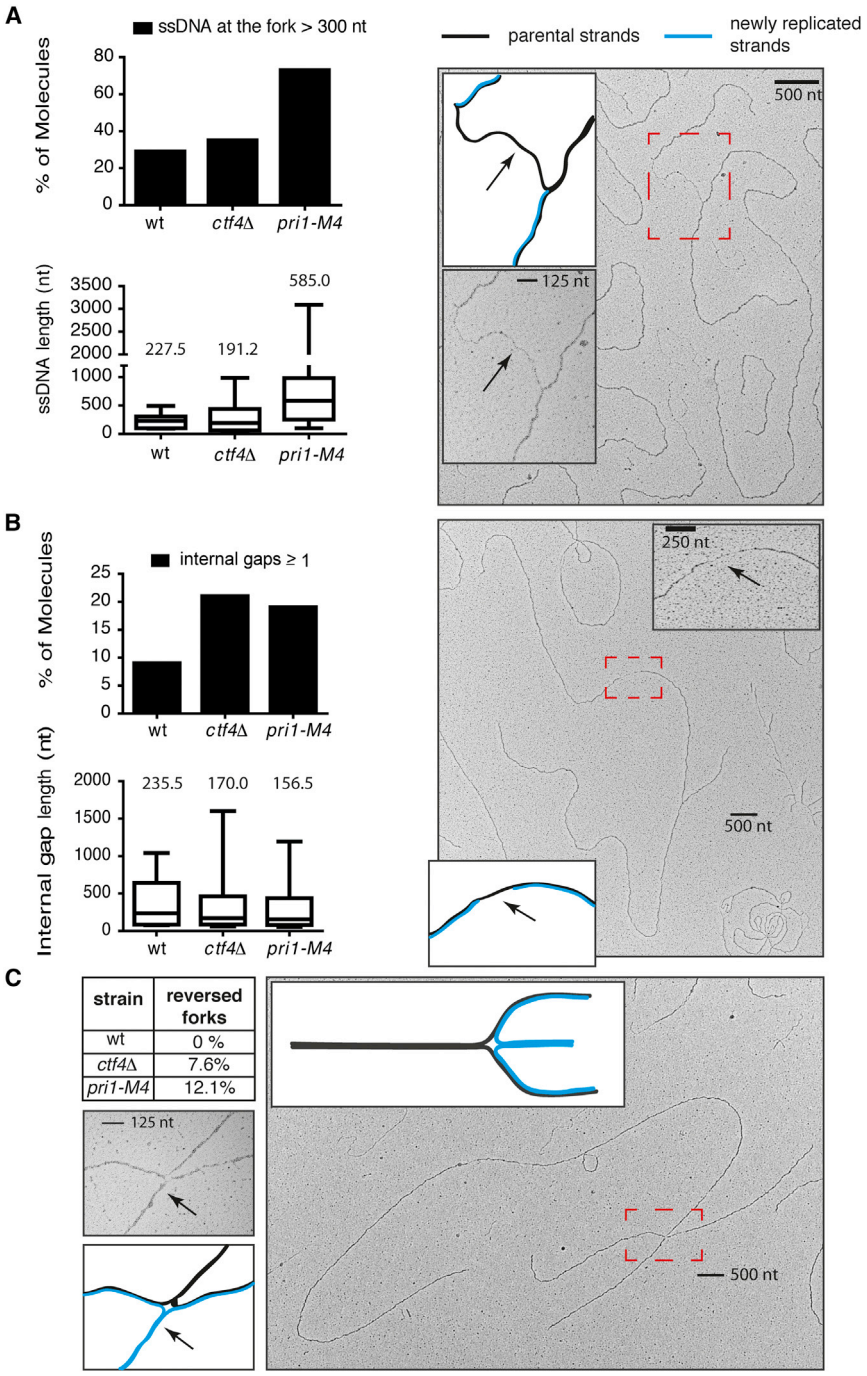


Figure 6. Structural Mapping of the Replication Defects in *ctf4Δ* and *pri1-M4*

(A–C) Electron microscopy analysis of the replication intermediates: (A) ssDNA stretches at the forks, (B) internal ssDNA gaps, and (C) reversed forks. WT (FY1000), *ctf4Δ* (HY2193), and *pri1-M4* (HY1607) cells were synchronized in nocodazole at 25°C prior to release at 30°C in MMS-containing medium (0.033%). Genomic DNA from cells collected at 90 min was psoralen crosslinked in vivo and enriched in RIs. Representative molecules, together with schematic representations, are shown. Quantifications of the indicated parameters are also displayed. Statistical analysis is based on ~80 replication forks for each strain. The analysis was conducted twice on genomic DNA derived from independent experiments with reproducible results.

Symington, 2004). 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, *ctf4*Δ, 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

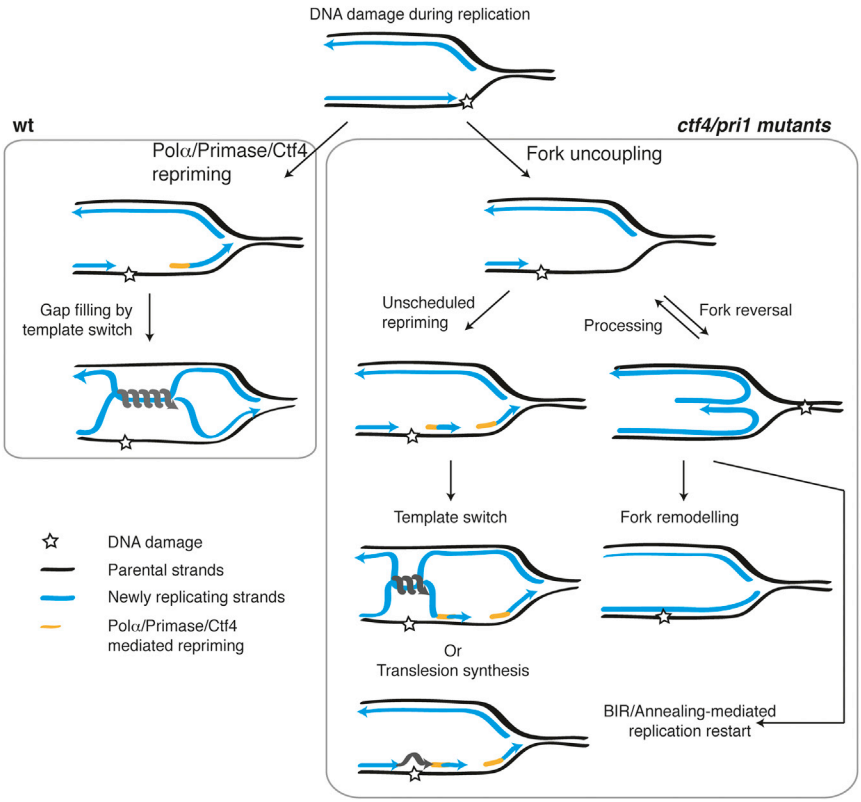


Figure 7. Hypothetical Model of DDT Orchestration in WT Cells versus Primase/Ctf4 Complex Mutants

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.

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 *sgs1Δ* 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 DDT/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 DDT. 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 (Figures 1A and 2B). The defects in error-free DDT were coincident with an increase in mutagenic DDT 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 DDT as the most prominent pathway of DDT, 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 *ctf4*Δ 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 (Bermejo 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., re-priming), 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; Doksani et al., 2009; Neelsen et al., 2013; Szakal and Branzei, 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 *ctf4*Δ and *pri1-M4* mutants display synthetic lethality with deletions of *RAD52* and *RAD59*, generally required for BIR, but not with *RAD51*, 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 neurological and developmental 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.

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