Visualization of recombination-mediated damage bypass by template switching

Michele Giannattasio\textsuperscript{1,2}, Katharina Zwicky\textsuperscript{3}, Cindy Follonier\textsuperscript{3,4}, Marco Foiani\textsuperscript{1,2}, Massimo Lopes\textsuperscript{3} and Dana Branzei\textsuperscript{1}

Template switching (TS) mediates damage bypass via a recombination-related mechanism involving PCNA polyubiquitination and polymerase 𝜈-dependent DNA synthesis. Using two-dimensional gel electrophoresis and EM, here we characterize TS intermediates arising in \textit{Saccharomyces cerevisiae} at a defined chromosome locus, identifying five major families of intermediates. Single-stranded DNA gaps of 150–200 nt, and not DNA ends, initiate TS by strand invasion. This causes reannealing of the parental strands and exposure of the nondamaged newly synthesized chromatid, which serves as a replication template for the other blocked nascent strand. Structures resembling double Holliday junctions, postulated to be central double-strand break–repair intermediates but so far visualized only in meiosis, mediate late stages of TS before being processed to hemicatenanes. Our results reveal the DNA transitions accounting for recombination-mediated DNA-damage tolerance in mitotic cells and replication under conditions of genotoxic stress.

DNA lesions compromise DNA replication and potentially lead to chromosome alterations and rearrangements\textsuperscript{4,6,14}. DNA-damage tolerance (DDT) mechanisms uphold genome integrity by ensuring replication completion via fork recovery and gap filling\textsuperscript{1}. Two distinct DDT modes are used in all organisms: an error-prone mode, largely accountable for mutagenesis and involving translesion synthesis\textsuperscript{3}, and an error-free recombination mode, known as TS, in which one newly synthesized strand serves as a replication template for the other blocked nascent strand\textsuperscript{4–7}. Proliferating cell nuclear antigen (PCNA) mono- and polyubiquitination are key regulators of DDT\textsuperscript{8}, promoting translesion synthesis\textsuperscript{9} and mediating TS\textsuperscript{10–13}, respectively.

The molecular mechanism of TS is only partially understood. Studies in yeast and mammalian cells have provided evidence that discontinuities opposite the lesions are created in the newly synthesized strands during replication in the presence of DNA damage\textsuperscript{5,6,14}. Repriming can reactivate transiently stalled forks \textit{in vitro}\textsuperscript{15}, thus potentially providing a molecular explanation for the formation of gaps. Pulse labeling experiments have indicated that the gaps are then filled in with the newly replicated strand used as a template\textsuperscript{6}. This notion has been substantiated by various genetic and molecular studies that further identified the Rad5 pathway, involving PCNA polyubiquitination as well as homologous-recombination activities, to be required in this process\textsuperscript{4,5,13,16}. Finally, X-shaped structures involving sister-chromatid junctions and having the genetic dependencies of TS intermediates have been identified with two-dimensional (2D) gel electrophoresis during replication of damaged templates\textsuperscript{7}. With this readout for TS, it has been shown that a replication step mediated specifically by polymerase 𝜈 is required\textsuperscript{17}, in congruence with other observations\textsuperscript{6,18,19}.

The RecQ helicase Sgs1 (BLM in mammalian cells), which is mutated in the cancer-prone Bloom syndrome, participates in error-free DDT as part of the Sgs1–Top3–Rmi1 (STR) complex, dissolving the TS intermediates\textsuperscript{7,19,20}. Persistent TS structures can also be resolved by the Mus81–Mms4 nuclease or by overexpressed bacterial or human resolvases\textsuperscript{21–23}. However, because all these enzymes can process a variety of substrates \textit{in vitro}\textsuperscript{24,25}, the structural nature of TS intermediates has remained undefined. Two main models of TS—strand invasion by a recombination-primed mechanism or annealing-mediated fork regression—have proposed that the intermediates are DNA structures in which the sister chromatids are paired\textsuperscript{2,6}. \textit{In vitro} biochemical characterization of known TS factors has provided evidence for both strand-invasion and fork-regression mechanisms\textsuperscript{27–29}, and single, double and nicked Holliday junctions (HJs) as well as hemicatenane-like structures have been proposed as potential intermediates\textsuperscript{1,17,20,23,30}.

Here we set out to identify the DNA structures and transitions involved in error-free DDT by TS, by using a combination of 2D gel electrophoresis and EM studies. This question is biologically important for several reasons. First, TS prevents incorporation of mutations\textsuperscript{1,3,8,31}, which ultimately lead to genome instability and cancer\textsuperscript{32}. Indeed, various TS factors function as tumor suppressors\textsuperscript{1,33}. Second, DNA-damaging agents, such as ultraviolet light, tobacco smoke and chemotherapeutics, all of which are potent damage inducers that occasionally target DDT pathways\textsuperscript{6,19,34,35}, cause specific mutational signatures associated with genome instability\textsuperscript{32}. Third, single-stranded DNA (ssDNA) gaps, as opposed to double-strand breaks (DSBs) in meiosis, have been suggested to be the predominant driver of recombination in mitotic cells\textsuperscript{31,36–38}, but the molecular mechanism remains elusive.

\textsuperscript{1}Istituto Fondazione Italiana per la Ricerca sul Cancro (FIRC) di Oncologia Molecolare (IFOM), Milan, Italy. \textsuperscript{2}Dipartimento di Bioscienze, Università degli Studi di Milano, Milan, Italy. \textsuperscript{3}Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland. \textsuperscript{4}Present address: Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA. Correspondence should be addressed to D.B. (dana.branzei@ifom.eu).

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RESULTS

A linear minichromosome system to study TS-mediated DDT

To obtain a homogeneous population of TS intermediates for EM studies, we used a 2µ-based 10.9-kb high-copy-number minichromosome, YLpFAT7.1 (Supplementary Fig. 1a)39. In S. cerevisiae, YLpFAT7.1 minichromosomes are maintained as linear fragments and are not entangled in concatemers (Supplementary Fig. 1b). To improve the specificity toward the intermediates of interest, we designed a restriction-enzyme mix that reduces the average length of the endogenous genomic DNA to less than 5 kb (Supplementary Fig. 1c). We synchronized minichromosome-containing wild-type and sgs1Δ cells and then allowed them to replicate in the absence or presence of the DNA-alkylating agent methyl methanesulfonate (MMS) (Fig. 1a). Similarly to endogenous chromosome replication, besides forming the usual intermediates such as replication forks and bubbles, minichromosome replication in the presence of MMS induced the formation of X-shaped DNA structures, which further accumulated in sgs1Δ cells, proximally to the 2µ replication origin (Fig. 1a and Supplementary Fig. 1a). The migration pattern of these joint molecules (JMs) appeared as two prominent spots on the X arc when we performed psoralen cross-linking in vivo before genomic extraction, instead of the smooth X-arc signal observed in the absence of psoralen cross-linking (Fig. 1b). TS intermediates arising on endogenous chromosomes showed the same migration pattern (Fig. 1b). These migration patterns are probably due to the psoralen cross-linking’s preventing migration of the junction point along the restriction fragment, thus stabilizing the initial X-shaped intermediates into specific spots40. Importantly, we found that the minichromosome-derived JMs shared the same genetic requirements with classical TS intermediates 7,12; that is, their formation depended on the Rad51 and Rad5 pathways in both wild-type and sgs1Δ backgrounds (Fig. 1c).

Isolation and assignment of TS intermediates

To visualize TS intermediates by EM, we psoralen cross-linked in vivo genomic DNA of wild-type and sgs1Δ cells enriched for minichromosome-derived DNA by cleavage of endogenous chromosomes (Supplementary Fig. 1c), and we further digested with HindIII before running preparative 2D gels (Fig. 2a). We then excised the X-shaped signal from the ethidium bromide–stained second-dimension gels (Fig. 2a), purified the DNA by electrophoresis and confirmed the migration pattern as branched molecules via a subsequent 2D gel (Fig. 2b).

We proceeded with EM analysis of DNA samples obtained from two independent experiments. Because we observed similar trends for all the crucial parameters described hereafter, we pooled the JMs analyzed in the results presented. We identified over 300 JMs for wild-type and sgs1Δ cells (Supplementary Fig. 1d). To ensure strict analysis of minichromosome-derived structures, we measured the lengths for all the initially identified JMs (n = 649). The assignment procedure, which allowed a 10% tolerance range in measurements, considered that minichromosome-derived TS intermediates should represent two HindIII 5.8-kb interconnected fragments, and the branches, taken to two to two, should be the size of one HindIII fragment41. In this way, we unequivocally assigned 43% (wild type) and 51% (sgs1Δ) of the initially identified X molecules as minichromosome-derived intermediates (Supplementary Fig. 1d). The JMs excluded by this assignment procedure are probably broken minichromosome-derived DNA or endogenous genomic DNA contaminants caused by partial digestion. We further classified the assigned minichromosome-derived JMs into symmetric molecules (in which the junction point was stabilized by homologous pairing, and two-to-two or all four branches had the same length) or asymmetric molecules (with junction points not stabilized by homologous pairing and all four branches of different length or only two equal arms).

Structural and genetic features of symmetric JMs

We classified these intermediates into five prominent families, F1–F5, on the basis of the structural features of the identified symmetric

Figure 1 TS intermediates formed on YLpFAT7.1 minichromosomes. (a) Genomic DNA samples isolated from wild-type (WT, CY11340) and sgs1Δ (CY11357) strains carrying YLpFAT7.1 minichromosomes and replicating in the presence of MMS 0.033%, analyzed by 2D gel electrophoresis with a minichromosome-specific probe, leu2d. Fluorescence-activated cell sorting (FACS) plots are shown adjacent to the gels. Exp, exponentially growing cells; α, α factor–arrested cells. The schematic represents the main replication intermediates and their shapes. (b) Genomic DNA samples from wild-type (CY11340) and sgs1Δ (CY11357) strains carrying YLpFAT7.1 and from the corresponding strains without minichromosomes, wild-type (CY12486) and sgs1Δ (CY13249), analyzed by 2D gel electrophoresis with probes for the minichromosome (leu2d) or the early origin of replication on endogenous chromosome III (ARS305). (c) 2D gels showing the genetic dependency of minichromosome-derived X molecules, analyzed in wild-type (CY11340), sgs1Δ (CY11357), rad5Δ (CY12388), rad5Δ (CY12777), sgs1Δ rad5Δ (CY12390) and sgs1Δ rad5Δ (CY12775).
JMs, which accounted for 84% of the JMs (Table 1). Besides the F1–F5 families, a small group of single Holliday junction (sHJ)-like structures (8/306) were present (Table 2). We also classified the asymmetric JMs into two families, F1* and F5* (Table 2).

We noted that two of the identified symmetric families were characterized by the presence of an ssDNA region proximal to the junction point on one or two of the four branches composing the JM. Because of the wealth of genetic and molecular information associating TS with gap filling, we reasoned that the early intermediates would be likely to contain such discontinuities close to the junction point. We called these families F1 and F2 (Table 1). The junction point in F1 resembled a cross between two linear double-stranded DNA (dsDNA) filaments, and there was an ssDNA stretch on one branch immediately proximal to the junction point (Fig. 3a and Supplementary Fig. 2), generally of 150–200 nt in length (Supplementary Fig. 1e). This family of molecules was equally distributed in both wild-type and sgs1Δ cells (Table 2 and Fig. 3c). The junction point in the F2 family involved a longer homology region and contained a thick DNA filament (Table 1 and Fig. 3b). Notably, F2 molecules contained a stretch of ssDNA on one or two branches of the JM close to the junction point (Fig. 3b and Supplementary Fig. 3) and were four times more prominent in sgs1Δ than in wild type (Fig. 3c). We examined how the F1 and F2 families were related by measuring the distribution of ssDNA length and other parameters (Fig. 3d). The median value of the ssDNA regions in F2 molecules (considering all molecules identified in both wild type and sgs1Δ) was 104 nt, shorter than the median of the ssDNA region of F1 molecules (196 nt) (Fig. 3d). Notably, the median value of the sum of the ssDNA regions and the thick DNA filament present at the junction point in F2 molecules (195 nt) was very similar, in the 10% tolerance range, to the median value of ssDNA regions found in F1 molecules (196 nt) (Fig. 3d). When we used the average values for the parameters above, instead of the median values, the same pattern emerged (Fig. 3d). Moreover, the same was true when we performed the above calculations for only the F1 and F2 molecules found in sgs1Δ (Fig. 3c). (The number of F2 molecules in wild type was too low compared to the number of F1 molecules in the same background to make such analysis meaningful; data not shown.) These results indicate that the F2 molecules derive from F1 by partial annealing of the gap region into the homologous duplex, leading to a double Y–like DNA structure (Fig. 3c).

The other symmetric families that we identified did not contain ssDNA discontinuities on the branches (Table 1). The F3 family structurally resembled double HJs (dHJs). The junction point was similar to a bubble, and it contained two dsDNA-like filaments of roughly the same length connecting the two HJ-like crosses, occasionally seen as rhomboid structures with an open center at the crossover position (Fig. 4a and Supplementary Fig. 4). The distance between the two HJ-like centers generally ranged from 0.15 to 0.6 kb but occasionally was longer (Fig. 4e). We also found molecules having features of both F2 and F3, which we denote F3*. The junction point in F3* still resembled a bubble, but we could always identify an ssDNA discontinuity on one of the two dsDNA filaments at the junction point (Supplementary Fig. 5), which probably indicates ongoing DNA synthesis on one of the strands. From the length of the duplex filaments at the F3 junction (Fig. 4e) and the ssDNA length in F1 molecules (Fig. 3d), we inferred that the DNA synthesis step implicated in TS6,17,19 often proceeds beyond the initial size of the gap, concomitantly leading to more extensive annealing of the parental strands. (Because two DNA duplexes are present between the crossover junctions in F3 and one duplex contains the newly replicated strands, it follows that the

Table 1 Families of symmetric TS intermediates

| Family | Schematic representation | Structural features | Described in |
|--------|--------------------------|---------------------|--------------|
| F1     |                         | • Simple cross       | Fig. 3, Supplementary Fig. 2 |
|        |                         | • ssDNA gap proximal to the junction point |              |
| F2     |                         | • Double Y– or dHJ-like structure | Fig. 3, Supplementary Fig. 3 |
|        |                         | • ssDNA gap on one or two branches proximal to the junction point |              |
|        |                         | • Thick DNA filament (1) at the junction point |              |
| F3     |                         | • dHJ-like structure | Fig. 4, Supplementary Fig. 4 |
|        |                         | • Two dsDNA filaments (1 and 2) of the same length connecting the two HJ-like crosses |              |
| F4     |                         | • Double Y– or dHJ-like structure | Fig. 4, Supplementary Fig. 6 |
|        |                         | • Thick DNA filament (1) at the junction point |              |
| F5     |                         | • Simple cross       | Fig. 4, Supplementary Fig. 7 |
|        |                         | • No ssDNA discontinuities proximal to the junction point |              |
other duplex must contain the paired parental strands, a notion for which we provide further evidence below.)

F4 resembles double-Y structures or dHJs42 (Table 1, and the junction point contains a small, almost closed bubble (Fig. 4b and Supplementary Fig. 6). A DNA filament, generally around 80 nt, of a thickness greater than that of a typical dsDNA filament and probably composed of two dsDNA filaments, was present at the junction point (Fig. 4b,e).

The F5 molecules resemble a cross between two dsDNA filaments (Table 1, Fig. 4c and Supplementary Fig. 7). Occasionally, at the junction point (and not on the branches as in F1) we detected ssDNA filaments that appeared to connect the two DNA duplexes implicated in the junction (Supplementary Fig. 7c).

We observed a few symmetric JMs with structural features of sHJs (8/306, Table 2 and Supplementary Fig. 8a), in addition to the F1–F5 families. We suspect that they represent the products of recombination events triggered by occasional nicks arising during replication or other families. We suspect that they represent the products of recombination events triggered by occasional nicks arising during replication or

Because the symmetric families structurally resembled HJs, we set out to investigate their biochemical properties. Incubation at high temperature causes JMs to branch migrate and to be resolved into linear DNA when the branch points reach the ends42–44. However, branch migration of canonical HJs, such as synthetic HJs and meiotic-recombination intermediates containing HJs, is prevented by the addition of magnesium cations45, which stabilize HJs in a stacked-X configuration45,46. We found that the minichromosome-derived TS intermediates arising in both wild-type and sgs1A cells showed comparable branch-migration activity regardless of the presence of magnesium cations in the buffer (Fig. 5a). Because of this feature, also previously reported for TS intermediates formed on endogenous chromosomes20,30, we conclude that the majority of TS intermediates are not canonical HJs.

Notably, we observed the same families of DNA structures for both the symmetric and the asymmetric junctions (described below) in sgs1A and wild-type cells (Table 2). Because sgs1A samples were specifically enriched in F2–F4 families (Table 2, Figs. 3c and 4d and Supplementary Fig. 1f), we conclude that these types of DNA structures are substrates for Sgs1 in vivo.

Figure 3 Representative EM pictures of F1 and F2 families. The analyzed molecules derive from two independent experiments. (a,b) Total views of the indicated X-shaped DNA structures and enlarged views with a schematic representation of the junction point (ssDNA regions in red and dsDNA regions in black). The branch sizes until the junction point are reported in kilobases. Equal arms are labeled with open circles and black squares, respectively. A schematic representation of the junction point is also shown, in which the two DNA duplexes that contribute to the junction are black and light gray. The black arrow in b marks the thick DNA filament at the junction point that we infer to be three-stranded. Scale bars are shown. (c) Chart showing the number of F1 and F2 molecules identified in wild-type and sgs1A cells. (d) Box plot of the described parameters. Center line, median; box limits, 25th and 75th percentiles; whiskers 10th and 90th percentiles; black dots, outliers; n, number of samples (molecules) in the data set. The median (M) and average (A) values are also shown. *P < 0.05 by two-tailed t test; NS, not significant. (e) Schematic representation of the F1-to-F2 transition. Black, parental strands; blue, newly synthesized strands; white triangle, hypothetical bulky DNA lesion.

Table 2 Percentage and number of JM families in wild-type and sgs1A cells

| Strains and JM families | F1* | F1  | F2  | F3* | F3  | F4  | F5  | F5* | sHJ | Total |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|
| Wild type              | 7.3% (11) | 15.9% (24) | 3.3% (5) | 0.7% (1) | 4.6% (7) | 8.6% (13) | 40.4% (61) | 15.2% (23) | 4% (6) | 100% (151) |
| sgs1A                  | 2.6% (4) | 14.8% (23) | 13.5% (21) | 5.8% (9) | 15.5% (24) | 18.7% (29) | 20.6% (32) | 7% (11) | 1.3% (2) | 99.8% (155) |

Percentages are shown with numbers of JMs in parentheses.

Table 1 Percentages of parental strands and newly synthesized strands in wild-type, sgs1Δ, and sgs1Δ cells

| Strains and JM families | F1  | F2  | F3  | F4  | F5  | sHJ | Total |
|------------------------|-----|-----|-----|-----|-----|-----|-------|
| Wild type              | 5.8% (9) | 15.5% (24) | 18.7% (29) | 20.6% (32) | 7% (11) | 1.3% (2) | 99.8% (155) |
| sgs1A                  | 2.6% (4) | 14.8% (23) | 13.5% (21) | 5.8% (9) | 15.5% (24) | 18.7% (29) | 20.6% (32) | 7% (11) | 1.3% (2) | 99.8% (155) |

Percentages are shown with numbers of JMs in parentheses.
Structural and genetic features of asymmetric JMs

Besides the symmetric molecules, 16% of junctions were asymmetric (49/306). We could divide them into two structural categories. The JMs of one of these categories, named F5* hereafter, had four branches of different length and no ssDNA discontinuities on the branches, and the junction points either resembled crosses between two linear DNA fragments or assumed more-complex structures in which three ssDNA filaments forming a triangle-like structure were visible (Fig. 5b and Supplementary Fig. 8b). Because the junction point was not stabilized by homologous pairing, we could rule out F5* being shJs, congruently with the results of the branch migration assays (Fig. 5a). Structurally, F5* molecules resembled hemicatenanes and the symmetric F5 molecules. The relative percentage of F5 and F5* versus other JMs was increased in wild-type versus required for the last step of hemicatenane resolution47. Moreover, the length of the dsDNA filaments visible at the F3 and F4 junction points is reported in kilobases. Scale bars are shown. (d) Chart showing the number of F3* and F3 (F3*/F3), F4 and F5 molecules identified in wild-type and sgs1Δ cells. (e) Box plot showing the length distribution of the dsDNA filaments at the junction points of F3*/F3 and F4 molecules. Center line, median; box limits, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; dots, outliers; n, number of samples (molecules) in the data set. The median (M) and average (A) values are also shown. *P < 0.05 by two-tailed t test.

We note that a subset of the F5* molecules (6/23 and 5/11 in wild type and sgs1Δ, respectively, 11/306 of the total JMs) had two branches of roughly the same length and therefore might represent reversed forks. The relative scarcity of JMs with structural properties compatible with those of reversed forks (less than 4% of the JMs) found here, and their absence in previous EM studies14, largely support the postreplicative gap-filling model of TS6,7,17,19,35.

The other category of identified asymmetric JMs structurally resembled both hemicatenanes and F1 molecules in that they contain an ssDNA discontinuity, of about 150–200 nt, on one of the branches, proximal to the junction point (Fig. 5c and Supplementary Fig. 8c). We labeled these intermediates F1* to illustrate the possibility, inferred from their structural features, of their being F1 precursors. We suggest that replication-associated hemicatenanes44 may become stabilized in symmetric junctions (F1) when encountering an ssDNA gap38,49, thus facilitating strand invasion of the gap region into the homologous sister duplex48.

Pseudo-dHJs mediate TS
dHJ-like structures of the F3 and F4 families could arise during gap filling if, besides the plećtonemic pairing of the newly synthesized DNA filaments, the parental strands also anneal in vivo (paranemic pairing, Fig. 6b). To analyze whether each of the dsDNA filaments present at the junction point in F3 molecules contain two ssDNA filaments paired in vivo (as opposed to their being together because of occasional nicking induced by the spreading procedure), we analyzed similarly isolated JMs under denaturing conditions. In this assay, large multiprotein complexes—mostly nucleosomal histones—tightly bound to DNA molecules protect the ssDNA filaments of DNA duplexes from being psoralen cross-linked, and, under conditions of denaturing spreading, their binding sites appear as strings of ssDNA bubbles connected by duplex regions representing the unbound linker...
dsDNA that was psoralen cross-linked\textsuperscript{50}. As a proof of principle, we identified several molecules with F3 dHJ features, whose examination revealed that the ssDNA strands composing each of the two duplexes present at the junction point in F3 molecules (Fig. 4a) are cross-linked and thus paired in vivo (Fig. 6a).

This type of structure is compatible with F3 molecules being canonical dHJs, in which the two dsDNA filaments connecting the two HJ crossoes are stabilized by plectonemic pairing (Fig. 6b, dHJ), or with a structure in which the parental ssDNA strands are connected to each other by only paranemic pairing, for instance by the action of Rad51 (Fig. 6b, pseudo-dHJ). We reasoned that the two types of structures could be distinguished if the chromosomal DNA were prepared without psoralen cross-linking in vivo: dHJs would structurally remain the same, whereas the paranemic pairing in pseudo-dHJ structures would be dissolved. This would expose the two parental ssDNA filaments, which upon treatment with mung bean nuclease should be digested to yield a double Y–like structure (Fig. 6b). Consistently with this latter prediction and resembling the behavior of TS intermediates arising on endogenous chromosomes\textsuperscript{30,36}, mung bean nuclease treatment of minichromosome-enriched DNA prepared in the absence of in vivo psoralen cross-linking led to a clear transition from the X arc to a double-Y structure (Fig. 6c). In contrast, the psoralen cross-linked samples were much more resistant in this regard (Fig. 6c). We propose, on the basis of these biochemical and structural features, that the F3 molecules accumulating in sgs1Δ and mediating TS are pseudo-dHJs.


discussion
HJs are the most discussed hypothetical intermediates for recombination; they have been modeled for DSB repair\textsuperscript{51} but to date have been visualized only in meiosis\textsuperscript{12}. Here we provide evidence that noncanonical HJs (pseudo-dHJs) are implicated in recombination-mediated gap filling by TS. Analysis of the structural features of early intermediates (F1 and F2) revealed that a substantial part of the gap region becomes paired with the homologous duplex containing the newly synthesized filament (evidence for paranemic pairing in Fig. 6), thus leading to a three-stranded DNA structure (F2, Figs. 3e and 7). The features of F2 molecules are consistent with a structure in which the gap region begins to be aligned with the homologous region of the sister duplex, before the onset of DNA synthesis (Figs. 3b,e and 7). Reannealing of the parental strands in F2 would facilitate the displacement and exposure of the nondamaged newly synthesized filament, which would then become available as a DNA-synthesis template for the stalled 3′ end proximal to the gap region (Figs. 3b and 7). Subsequent extension of the 3′ end would give rise to F3′-like molecules with ongoing DNA synthesis on only one filament of the F3 junction point (Supplementary Fig. 5 and Fig. 7). As annotated in the features of the observed symmetric structures, measurements of the ssDNA regions and of the thick DNA filaments present at the junction point in F1 and F2 molecules fully support this view (Fig. 3).

An alternative to the model and interpretation above is that the F2 molecules already illustrate DNA synthesis–mediated gap filling initiated by the invasion of the transiently stalled 3′ end into the homologous sister duplex and the formation of a D loop–like structure. A caveat with this interpretation is that parental-strand annealing is not necessarily expected to happen in this scenario, but it must be invoked to explain the subsequent paired parental strands observed in F3 and F4 molecules (Fig. 6a). Such pairing could happen if elongation of the D loop—which this model suggests to happen in F2 molecules—is coupled with annealing of the parental strands.
RecQL5 and BLM 55,56. We thus propose that both dHJ dissolution similarly to what was proposed for the mammalian RecQ helicases.

Table 2 compared to wild type (4.6%) cells (Fig. 7).

However, such an interpretation is incongruent with the presence of ssDNA filaments on both sides of the junction point in F2 molecules (Fig. 3b). Thus, the data support the gap-mediated model of strand invasion, which is also consistent with other previous genetic deductions.17,19,37,48,52

Regardless, however, of whether TS is initiated by the gap region or by the adjacent 3′ end close to the gap, completion of TS DNA synthesis can be followed by second-end capture, with formation of dHJ-like structures, or by strand displacement that would lead to linear molecules and noncrossover products. In agreement with the inference that F3 molecules represent dHJ-like molecules that are resolved by the STR complex,20,47,53,54, we found an increased percentage of F3 molecules in sgs1Δ (15.5%) as compared to wild type (4.6%) cells (Table 2 and Supplementary Fig. 1f). Besides F3 and F4 types, F2 and F3* families also accumulated in sgs1Δ samples (Table 2 and Supplementary Fig. 1f), thus revealing that Sgs1 also potentiates D-loop displacement (Fig. 7) similarly to what was proposed for the mammalian RecQ helicases RecQ5 and BLM55,56. We thus propose that both dHJ dissolution and strand displacement account for Sgs1 roles in suppressing sister chromatid exchanges and mitotic crossover54,57 and in facilitating TS-intermediate resolution52.

Dissolution of dHJ-like intermediates by STR is expected to ultimately lead to symmetric hemicatenanes or F5-like molecules (Fig. 7) later resolved by Top3. We detected, in addition to F5 structures, intermediates of the F3 dissolution process (F4, Figs. 4b and 7), which still accumulated in sgs1Δ cells (Table 2 and Fig. 4d). This indicates that the last step of dissolution is slow, probably because of topological constrains, and that Sgs1 facilitates Top3 activity also in this context58 (Fig. 7).

Previous work on factors that influence TS-intermediate resolution failed to reveal a role for factors such as Srs2 that are important for D-loop unwinding25,54,57. Why problems in this early step do not translate into X-molecule accumulation is not well understood. On the basis of these new results, it seems likely that a combination of events—redundancy of D-loop unwinding, very efficient pathways of second-end capture and subsequent dissolution of dHJ-like intermediates, as well as sensitivity limits of previously conducted 2D gel assays—is the underlying reason. In this new light, it also appears likely that the TS-intermediate accumulation in smc5, smc6 and mms21 mutants59,60 is an indication that SUMOylation mediated by Smc5–Smc6–Mms21 influences dHJ dissolution, possibly by facilitating STR activity or by enabling the formation of alternate Top3 complexes with similar in vivo activity to that of STR59,60. The method used here and the advances in the structural studies of replication and recombination intermediates promise to markedly contribute to unraveling how different genome-integrity pathways—which show similar phenotypic outcomes when

Figure 6 Observation of F3 molecules by denaturing spreading and biochemical identification of paranemic pairing. (a) Entire and enlarged view of the junction point of an F3 molecule after denaturing spreading. The analyzed molecules derive from two independent experiments. A schematic representation of the junction point is shown, with ssDNA regions in red and dsDNA filaments in black. The black arrow indicates a heavily cross-linked dsDNA filament that can be used as reference for the thickness of a dsDNA filament in this experimental condition. A schematic representation of the junction point showing the contribution of the ssDNA filaments from the two DNA duplexes (labeled in black and light gray) to the junction point is shown. Branch sizes until the junction point and the length of the DNA filaments in the junction point are also reported (in black) in kilobases. Scale bars are shown. (b) Schematic representations of the possible outcomes of the mung bean nuclease treatment on pseudo-dHJs or classical dHJs, with or without in vivo psoralen cross-linking. (c) Mung bean nuclease–treated and nontreated samples run on 2D gels in parallel. Samples are genomic DNA from sgs1Δ (CY11357) cells carrying the minichromosome YLPAT7.1 and replicating in the presence of MMS, extracted after psoralen cross-linking in vivo or without psoralen cross-linking. The experiments were conducted three times with qualitatively identical results.
individually ablated—are integrated to support basic chromosome functions such as DDT and replication completion.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

M.G. designed and executed the experiments, acquired the EM images, analyzed the data and made the figures. K.Z. and C.F. acquired a subset of EM images and helped with EM data analysis. M.F. conceived the project and communicated the results. M.L. conceived the project, supervised the EM part, analyzed the EM data and commented on the manuscript. D.B. conceived and supervised the project, designed the experiments, analyzed the data and wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture, psoralen cross-linking and genomic DNA extraction for 2D gels. Yeast strains used in this work are listed in Supplementary Table 1. The yeast strains carrying the minichromosome YLPAT7.1 (ref. 39) were grown in synthetic medium without leucine, arrested in G1 with alpha factor (Sigma, 4 µg/ml) and released in YPD medium or YPD medium containing 0.033% MMS. Cells were fixed in 0.1% sodium azide for 40 min on ice as described. In brief, for the in vivo psoralen cross-linking cellular pellets corresponding to 1 × 10⁹ to 2 × 10⁹ cells were washed and resuspended in 5 ml of sterile cold water. Four cycles of 10 min each of in vivo psoralen cross-linking were performed by placing the cell suspensions (transferred in 6-well plastic tissue-culture plates with flat bottoms) in a Stratagene Stratalinker 1800 irradiation chamber, under 365 nm (UV). Before each irradiation cycle, a fresh 0.3-ml aliquot of psoralen (4, 5′, 8-trimethyl-psoralen) (Sigma, 0.1 mg/ml dissolved in ethanol) was added to the cell suspension, mixed with cut micropipette tips and incubated for 5 min under aluminum foil. During the entire procedure, the plates containing the cell suspensions were placed on ice. After this procedure, the cell suspensions were transferred to 50-ml conical tubes, the pellets were washed twice with sterile cold water, and genomic DNA was extracted as previously described.

Preparative 2D gels, electroelution and electron microscopy. For preparative 2D gels, 50 to 100 µg of genomic DNA was digested with a restriction-enzyme mix containing PacI, XhoI, SgrAI, BseHI, SpeI, BclI, PmlI and HindIII, precipitated with potassium acetate/isopropanol and suspended in 1× TE or 10 mM Tris-HCl, pH 8. Digested genomic DNA was run on Owl Separation Systems Model A2 electrophoresis chambers (gel tray 27 × 20 cm; 2.5 L buffer) with nonautoclaved 1× TBE and agarose gels of 500 ml. The first dimensions (0.35% agarose gels) were run at 50 V for 24 h at room temperature, and the second dimensions (0.9% agarose gels) for 12 h at 150 V (current limited at 150 mA) at 4 °C. For electroelution, agarose fragments were run in 1× TBE for 5 h at 100 V at room temperature in an Elutrap chamber (Bio-Rad) with BT-1 and BT-2 membranes according to the manufacturer’s instructions. Recovered DNA solutions (around 0.8 ml) were concentrated on conical Amicon Ultra centrifugal filters (0.5 ml; MWCO 100 kDa) according to the manufacturer’s instructions. DNA samples from the electroelution were subjected to DNA spreading on carbon-coated metal grids (4-nm thickness) in the presence of uranyl acetate, and this was followed by platinum-based rotary shadowing (0.4 nm without rotation and up to 8 nm with rotation) and EM analysis as previously described.

Branch migration and mung bean nuclease digestion assays. Branch migration and mung bean nuclease digestion assays were conducted as previously described. For branch migration assays, the first-dimension slices were incubated in TNE buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8, and 0.1 mM EDTA, pH 8) or TNM buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8, 10 mM MgCl₂, and 0.1 mM EDTA, pH 8), for 13 h at 65 °C. For mung bean nuclease assays, 12 µg of digested genomic DNA was precipitated with potassium acetate/isopropanol and resuspended in 1× mung bean nuclease buffer. 40 units of mung bean nuclease (New England Biolabs) were added to each sample, and this was followed by incubation for 75 min at 37 °C. DNA was precipitated again and resuspended in 10 mM Tris-HCl, pH 8, and the first- and second-dimension gels were run as described above.

Assignment procedure for ssDNA regions on the DNA molecules analyzed. The assignment criteria for an ssDNA region on the DNA molecules analyzed by the EM procedure used here was recently described. We note that in order to assign an 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 decreases to close to one half. The thickness of the DNA filament between these two points can vary slightly, owing to the positioning of the filament during the spreading procedure, but in general it is clearly lower than the average value of the thickness of a dsDNA filament, which in these experimental conditions is distributed around 20 Å. Although the difference between dsDNA and ssDNA is promptly detected, it is generally difficult to visually distinguish thickness differences in multistrand DNA (more than two strands). There are two technical explanations behind this. The first reason is the reduced relative difference in thickness, by a factor of 2 in the transition from single- to double-stranded DNA but a factor of only 1.5 from double- to triple-stranded DNA. The second reason relates to the DNA visualization procedure: it was also previously shown that the combination of uranyl acetate staining and platinum rotary shadowing used in this work is instrumental to appreciate the difference in thickness between dsDNA filaments and ssDNA filaments. This is because the platinum rotary shadowing stains only dsDNA, whereas the uranyl atoms positively stain both ssDNA and dsDNA. Thus, the observed difference in thickness of the stained molecules—largely determined by the amount of deposited heavy atoms—generally exceeds the difference in thickness of the biological material itself. For the same reasons, three-stranded DNA (inferred to be present at the junction point in F2 molecules) will not be much more covered by heavy atoms than dsDNA would be, because the dsDNA is already abundantly recognized by both staining procedures, thus reducing the relative contribution of an additional DNA strand to the thickness.

Length measurements. The length measurements were performed with 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), obtained by the measurement in pixels of a plasmid molecule of known length used as internal standard, as detailed in ref. 41. We note that the length of the ssDNA stretches reported here, with the same conversion factors for both dsDNA and ssDNA, might have been underestimated by a maximum experimentally determined factor of 15%, calculated for extreme conditions of long and entirely single-stranded DNA molecules that show reduced anchorage to the carbon layer and have different stretching properties.

Assignment procedure for the identified joint molecules. The assignment procedure of JMs considers that the total size of the X molecules (the sum of the branches and, when applicable, of the extended homology region) must fall in the interval 10,584–12,953 bp, which corresponds to the size of two HindIII 5.88-kb linear fragments interconnected (Supplementary Fig. 1a and ref. 41). Moreover, the branches of an X molecule taken two to two, together with the extended homology region for molecules in the categories F2, F3 and F4, must have a length that falls in the interval 5,292–6,468 bp, which corresponds to the size of one HindIII fragment.