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Pervasive and essential roles of the Top3-Rmi1 decatenase orchestrate recombination and facilitate chromosome segregation in meiosis

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Summary

The Bloom's helicase ortholog, Sgs1, plays central roles to coordinate the formation and resolution of joint molecule intermediates (JMs) during meiotic recombination in budding yeast. Sgs1 can associate with type-I topoisomerase Top3 and its accessory factor Rmi1 to form a conserved complex best known for its unique ability to decatenate double-Holliday junctions. Contrary to expectations, we show that the strand-passage activity of Top3-Rmi1 is required for all known functions of Sgs1 in meiotic recombination, including channeling JMs into physiological crossover and noncrossover pathways, and suppression of non-allelic recombination. We infer that Sgs1 always functions in the context of the Sgs1-Top3-Rmi1 complex to regulate meiotic recombination. In addition, we reveal a distinct late role for Top3-Rmi1 in resolving recombination-dependent chromosome entanglements to allow segregation at anaphase. Surprisingly, Sgs1 does not share this essential role of Top3-Rmi1. These data reveal an essential and unexpectedly pervasive role for the Top3-Rmi1 decatenase during meiosis.

Introduction

DNA joint molecules (JMs) are central intermediates in chromosome repair by homologous recombination (Haber, 2013). A JM results from exchange of DNA strands between one or both ends of a broken chromosome and an intact homologous template chromosome. The invading strand(s) primes DNA synthesis to restore sequences that were lost or damaged at the site of the original lesion. Finally, JMs must be resolved into individual duplexes to allow chromosomes to separate during anaphase.

A number of distinct JM structures have been identified from yeast cells undergoing recombinational repair. These include canonical three and four armed structures such as D-loops or Single-End Invasions (SEIs), Holliday junctions (HJs), complex structures such as multi-chromatid JMs (mcJMs, comprising three and four interconnected DNAs),

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recombinant JMs (containing recombined DNA strands) and catenated structures that lack canonical HJs (Bzymek et al., 2010; Cromie et al., 2006; Hunter and Kleckner, 2001; Jessop and Lichten, 2008; Liberi et al., 2005; Lopes et al., 2003; Mankouri et al., 2011; Oh et al., 2007; Oh et al., 2008; Schwacha and Kleckner, 1995). This variety of JM structures and the different cellular contexts in which they form demands the regulated action of a variety of processing enzymes including DNA helicases, topoisomerases and endonucleases (Blanco et al., 2014; Castor et al., 2013; De Muyt, 2012; Eissler et al., 2014; Gallo-Fernandez et al., 2012; Hickson and Mankouri, 2011; Matos et al., 2011; Matos et al., 2013; Mimitou and Symington, 2009; Oh et al., 2007; Saugar et al., 2013; Schwartz and Heyer, 2011; Wyatt et al., 2013; Zakharyevich et al., 2012).

Regulation of JM processing is especially important during meiotic recombination where two biological imperatives must be achieved. First, each pair of homologous chromosomes must become connected by at least one crossover, which allows their bipolar orientation on the spindle and accurate disjunction at meiosis I (Hunter, 2006). Second, as hundreds of recombination events are induced during meiosis in most organisms, the ensuing JM resolution must be highly efficient to allow chromosomes to cleanly separate during anaphase (De Muyt et al., 2012; Jessop and Lichten, 2008; Oh et al., 2008; Zakharyevich et al., 2012).

Reliance on the various JM resolving pathways during meiosis differs between organisms (Bellendir and Sekelsky, 2013; Kohl and Sekelsky, 2013; Schwartz and Heyer, 2011). However, budding yeast, plants and mammals utilize largely identical pathways. In these organisms, a majority of crossovers arise via a pathway defined by the MutSγ complex (Msh4-Msh5), inferred to stabilize nascent JMs (Borner et al., 2004; Snowden et al., 2004), and a crossover-specific double-Holliday junction (dHJ) resolving factor, comprising the endonuclease MutLγ (Mlh1-Mlh3) and a nuclease-independent function of Exo1 (Nishant et al., 2008; Ranjha et al., 2014; Rogacheva et al., 2014; Zakharyevich et al., 2010; Zakharyevich et al., 2012).

Orthologs of the Bloom's helicase play a central role to orchestrate recombination during meiosis (Hartung et al., 2007; Holloway et al., 2010; Oh et al., 2007; Zakharyevich et al., 2012). Data from budding yeast show that Bloom’s ortholog Sgs1 facilitates the major physiological pathways of crossover and non-crossover formation by dissociating the products of promiscuous strand exchange. Without Sgs1, aberrant “off-pathway” joint molecules (including mcJMs) become prevalent, resolution by the structure-selective endonucleases (Mus81-Mms4, Slx1-Slx4 and Yen1) becomes predominant and unregulated recombination ensues (De Muyt et al., 2012; Jessop and Lichten, 2008; Oh et al., 2007; Oh et al., 2008; Zakharyevich et al., 2012).

Both Sgs1 and human BLM can interact with a single-strand decatenase, respectively Top3 and TOPIIIα (Gangloff et al., 1994; Johnson et al., 2000; Wu et al., 2000). Together with OB-fold proteins, Rmi1 and RMI1/2, these proteins assemble a conserved complex, Sgs1-Top3-Rmi1 (STR) in yeast and BLM-TOPIIIa-RMI1/2 (BTR) in human, best known for its unique ability to disassemble dHJs *in vitro* to produce exclusively non-crossover duplexes (an activity termed "dissolution" to distinguish it from endonuclease-mediated “resolution”)

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(Bussen et al., 2007; Cejka et al., 2010b; Singh et al., 2008; Wu et al., 2006; Wu and Hickson, 2003). Thus, STR comprises a potent anti-crossover activity important for genome stability.

In budding yeast, *top3*Δ and *rmi1*Δ null mutants are extremely slow growing, show high levels of genome instability, rapidly accumulate suppressors and fail to form tetrad asci (Chang et al., 2005; Gangloff et al., 1999; Gangloff et al., 1994; Mullen et al., 2005; Oakley et al., 2002; Shor et al., 2002; Wallis et al., 1989). For these reasons, the role of the Top3-Rmi1 decatenase in meiotic JM processing has remained unclear. The sole study of *TOP3* meiotic function in budding yeast showed that meiotic divisions fail in *top3*Δ null mutants (Gangloff et al., 1999). Importantly, this defect could be suppressed by preventing the initiation of recombination. A recombination dependent role for topoisiomerase 3 in meiotic progression was also inferred from studies in *C. elegans top-3* mutants (Wicky et al., 2004). Studies in *A. thaliana* have shed further light on the meiotic defects of both *top3*α and *rmi1* mutants, showing that homologous chromosomes were entangled at metaphase I and unable to separate at meiosis I (Chelysheva et al., 2008; Hartung et al., 2008). The molecular defects that underlie these phenotypes are unknown.

A major role for Top3-Rmi1 in meiotic JM processing has not been envisioned because most dHJs appear to be resolved specifically into crossovers via endonuclease-mediated resolution (Allers and Lichten, 2001; Zakharyevich et al., 2012). Moreover, noncrossovers are thought to arise via synthesis-dependent strand annealing, in which extended D-loops are simply unwound by a helicase such as Sgs1 (McMahill et al., 2007).

Here, we use two types of meiosis-specific conditional allele to discriminate distinct early and late roles for Top3-Rmi1 in regulating JM formation and resolving chromosome entanglements. Contrary to expectations, all meiotic roles previously defined for Sgs1 also require Top3-Rmi1 implying that STR generally acts as a functional module, with concerted helicase-decatenase activity, to regulate JM formation and limit the requirement for processing by the structure-selective endonucleases. Execution-point analysis, enabled by inducible degron alleles of Top3 and Rmi1, reveal an essential late role for Top3-Rmi1 in resolving recombination-dependent chromosome entanglements. Unlike the earlier functions of STR, Sgs1 is dispensable for this late role of the Top3-Rmi1 decatenase. Degron analysis further reveals cryptic late roles for Top3-Rmi1 in non-crossover formation and suppression of non-allelic (ectopic) crossing over.

Together, these data reveal an essential and unexpectedly ubiquitous role for a single-strand decatenase activity during meiosis. We infer that JMs have more complex structures than typically envisioned, containing entanglements and topological constraints that can only be resolved by Top3-Rmi1 mediated single-strand passage.

Results

All STR Components are Required to Suppress Aberrant Recombination During Meiosis

Slow growth of *top3*Δ and *rmi1*Δ null mutants is exacerbated in diploid cells and precludes large-scale temporal analysis of meiotic recombination. To overcome this impediment, we

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constructed strains carrying meiosis-specific shut-off alleles. In these strains, the *CLB2* promoter, which is strongly repressed during meiosis, replaces native promoters of *TOP3* and *RMI1* (Lee and Amon, 2003; Oh et al., 2008). DNA events of meiotic recombination were monitored using a series of Southern blot assays at the well-characterized *HIS4::LEU2* recombination hotspot (Figure 1A)(Hunter and Kleckner, 2001; Oh et al., 2007; Schwacha and Kleckner, 1995). The key advantage of these physical assays is that they allow recombination to be quantified in the entire, unbiased cell population and do not require cells to maintain viability following meiosis.

*Xho*I polymorphisms between the two parental *HIS4::LEU2* alleles produce DNA fragments diagnostic for DSBs, JMs and crossover products (Figures 1A; for JM analysis see Figure 2A, below). DSBs, allelic crossovers and aberrant non-allelic ("ectopic") crossovers were analyzed using one-dimensional gels (Figure 1A,B). Ectopic crossing-over involves exchange between *HIS4::LEU2* and sequences at the native *LEU2* locus located ~25kb away on the same chromosome (chr III)(Grushcow et al., 1999). Noncrossover products are detected by conversion of a *Bam*HI/*Ngo*MIV restriction-site polymorphism located directly at the site of DSB formation (Figure 1A,C).

Defects of *sgs1* single mutants are relatively cryptic: allelic crossovers and noncrossovers form at high levels and spore viability remains relatively high (Figure 1A-E; Figure S1)(De Muyt et al., 2012; Jessop et al., 2006; Oh et al., 2007; Zakharyevich et al., 2012). However, ectopic crossovers are increased 7-fold above wild-type levels (Figure 1E). Consistent with Sgs1 working as a complex with Top3-Rmi1 to suppress aberrant recombination, P_{CLB2}-*SGS1*, P_{CLB2}-*TOP3*, and P_{CLB2}-*RMI1* shut-off alleles conferred very similar phenotypes (Figure 1A-E). Timing and levels of DSBs were similar in all strains, allelic crossovers and noncrossovers formed at near wild-type levels, while ectopic recombination was elevated 6.5 fold.

We previously showed that *sgs1* mutation results in modest increases in genetic map distances due to an increase in closely-spaced double crossovers (Oh et al., 2007). A manifestation of this phenotype is reduced intensity of positive interference (the tendency for crossovers to be widely and evenly spaced). Using standard tetrad analysis to analyse recombination in an interval containing the *HIS4::LEU2* hotspot (Figure 1F), we showed that P_{CLB2}-*TOP3*, and P_{CLB2}-*RMI1* mutations have a similar effect (Figure 1G). Map distances of the *URA3–HIS4::LEU2* interval were increased 1.44, 1.65 and 1.40-fold in P_{CLB2}-*SGS1*, P_{CLB2}-*TOP3* and P_{CLB2}-*RMI1* strains, respectively (Figure 1G). Crossover interference was assessed by calculating the NPD ratio, the number of observed non-parental ditype tetrads (NPDs, diagnostic of 4-chromatid double crossovers within a single interval) divided by the number of NPDs expected in the absence of interference (Papazian, 1952). In wild-type tetrads, crossovers in the *URA3–HIS4::LEU2* interval showed strong positive interference as indicated by an NPD ratio of 0.21 ± 0.11 (Figure 1G). In P_{CLB2}-*SGS1* and P_{CLB2}-*RMI1* tetrads, NPD ratios were not different from one (0.86 ± 0.26 and 1.17 ± 0.40, respectively), indicating diminished interference in this interval. In P_{CLB2}-*TOP3* tetrads, the NPD ratio was 2.17 ± 0.57, suggestive of negative crossover interference (however, this was not significant with the current data set).

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Altered JM Spectra in P_{CLB2}-TOP3 and P_{CLB2}-RMI1 Mutants

In the *sgs1* mutant, the JM spectrum is altered to favor intersister JMs and mcJMs involving three or four chromatids (Oh et al., 2007). To analyze the effects of the P_{CLB2}-*TOP3 and* p_{CLB2}-*RMI1* alleles, JMs at *HIS4::LEU2* were analyzed using native/native two-dimensional (2D) gels, which reveal the branched character of these intermediates and allow their accurate quantification (Figure 2A,B)(Bell and Byers, 1983; Hunter and Kleckner, 2001; Oh et al., 2007; Schwacha and Kleckner, 1995). Similar to P_{CLB2}-*SGS1*, the P_{CLB2}-*TOP3* and p_{CLB2}-*RMI1* alleles cause elevated levels of both mcJMs and, to a lesser extent, intersister JMs (Figure 2C,D). In addition, disappearance of JMs is generally delayed relative to wild type.

To avoid changes in JM spectra caused by variable resolution, JM analysis was also performed in the *ndt80* background, which arrests in prophase I with unresolved JMs (Allers and Lichten, 2001). These experiments confirmed that levels of mcJMs and intersister JMs are increased by the P_{CLB2}-*SGS1*, the P_{CLB2}-*TOP3* and p_{CLB2}-*RMI1* mutations (Figure 2E,F). Increased JM formation between sister chromatids is reflected in a reduced ratio of interhomolog-dHJs to intersister JMs (IH/IS ratio), which shifts from ~5 in wild type to ~2 in all three STR mutants (Figure 2D). JMs that migrate at the same positions as Single-End Invasions, but accumulate in the *ndt80* mutant, also appear to be elevated in STR mutants (Figure 2D). These structures are likely heterogenous D-loop like species and their elevation is consistent with the inference that processing of early intermediates is defective in STR mutants.

Crossovers and Noncrossovers in P_{CLB2}-TOP3 and P_{CLB2}-RMI1 Mutants Are Dependent on the Structure-Selective Endonucleases

Studies from the Lichten group previously showed that noncrossovers formed ~35 minutes earlier than crossovers and that this differential timing was lost in *sgs1* mutants (Allers and Lichten, 2001; De Muyt et al., 2012)(see below). Analogously, noncrossovers at the *HIS4::LEU2* locus appear ~40 minutes earlier than crossovers in wild-type cells, while in P_{CLB2}-*SGS1* cells this difference was reduced to ~8 minutes (Figure 3A and data not shown). Like P_{CLB2}-*SGS1*, the P_{CLB2}-*TOP3*, and P_{CLB2}-*RMI1* alleles diminish the differential timing of crossovers and noncrossovers to ~9 minutes.

The central role of Sgs1 in meiotic JM processing is starkly revealed by the observation that the high levels of crossovers and noncrossovers formed in *sgs1* mutant cells are almost completely dependent on the Mus81-Mms4, Slx1-Slx4 and Yen1 endonucleases, which play minor roles in wild-type meiosis (De Muyt et al., 2012; Zakharyevich et al., 2012). The loss of early noncrossover formation seen in *sgs1* mutants (Figure 3A) is a manifestation of this shift, which necessitates post-pachytene activation of endonuclease-dependent resolution via Ndt80-dependent expression of polo-like kinase, Cdc5/Plk1 (De Muyt et al., 2012; Matos et al., 2011; Sourirajan and Lichten, 2008). Consistently, noncrossovers are greatly diminished in P_{CLB2}-*SGS1 ndt80*Δ cells relative to an *ndt80*Δ single mutant (Figure 3B,C). Analogous decreases in noncrossover formation are seen for both P_{CLB2}-*TOP3 ndt80*Δ and P_{CLB2}-*RMI1 ndt80*Δ strains (Figure 3B,C).

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Consistent with the inference that Sgs1 always works together with Top3-Rmi1, both crossovers and noncrossovers are diminished in quadruple mutants carrying either P_{CLB2}-*TOP3* or P_{CLB2}-*RMI1* shut-off alleles together with mutations in the three JM resolving endonucleases (P_{CLB2}-*MMS4 slx4*Δ *yen1*Δ; Figure 3D,E). This synthetic defect matches that of the P_{CLB2}-*SGS1* P_{CLB2}-*MMS4 slx4*Δ *yen1* quadruple mutant, and contrasts the high levels of crossovers and noncrossovers seen in P_{CLB2}-*SGS1*, P_{CLB2}-*TOP3* and P_{CLB2}-*RMI1* single mutants (Figure 1E), and the P_{CLB2}-*MMS4 slx4*Δ *yen1*Δ triple mutant (Figure 3D,E).

Consonant with the severe reduction of recombinant products, 2D gel analysis shows that unresolved JMs persist in all three STR + resolvase quadruple mutants (Figure 3D,E). As shown previously (Zakharyevich et al., 2012), P_{CLB2}-*SGS1* single mutants and the P_{CLB2}-*MMS4 slx4*Δ *yen1*Δ triple mutant are relatively proficient for JM resolution, while the corresponding quadruple mutant shows high levels of unresolved JMs even at very late times in meiosis (Figure 3D,E). Similarly, high levels of JMs persist in quadruple mutants with either P_{CLB2}-*TOP3* or P_{CLB2}-*RMI1* alleles (Figure 3D,E).

Mutation of *SGS1* was shown to suppress the low levels of JMs and crossovers caused by mutations in a number of pro-crossover factors, called the ZMM or SIC proteins (Jessop et al., 2006; Oh et al., 2007). We found that this phenotype is also shared by the P_{CLB2}-*TOP3* (Figure S2; P_{CLB2}-*RMI1* was not tested).

Collectively, these data indicate that Sgs1, Top3 and Rmi1 likely function as a complex to prevent aberrant JM formation, limiting alternative processing by the Mus81-Mms4, Yen1 and Slx1-Slx4 endonucleases, and thereby promoting the major crossover and noncrossover pathways. Consistently, analysis of double mutants, P_{CLB2}-*TOP3* P_{CLB2}-*SGS1* and P_{CLB2}-*RMI1* P_{CLB2}-*SGS1*, indicated that STR mutants are epistatic for meiotic recombination phenotypes (Figure S3).

Sgs1-Top3 Interaction and Sgs1 Helicase Activity are Required to Suppress Aberrant Recombination During Meiosis

To obtain evidence that direct interaction between Sgs1 and Top3 is important for regulating meiotic recombination, we also analyzed cells carrying the *sgs1*-Δ*N82* allele, which lacks residues essential for interaction with Top3 and mimics the *top3* mutant phenotype (Weinstein and Rothstein, 2008)(to circumvent slow growth caused by *sgs1*-Δ*N82*, cells were made heterozygous with P_{CLB2}-*SGS1*). Meiotic phenotypes of *sgs1*-Δ*N82* cells were indistinguishable from those conferred by P_{CLB2} alleles of the STR genes, including elevated ectopic recombination and altered timing of crossovers relative to noncrossovers (Figure S4).

Biochemical analysis of STR-mediated decatenation showed that Sgs1 helicase activity is required to form singled-stranded DNA for Top3 cleavage (Cejka et al., 2012). Notably, when single-stranded DNA was provided in the substrates, Sgs1 still catalyzed decatenation but did so independently of its helicase activity. *In vivo* studies support the inference that Sgs1 can facilitate Top3-Rmi1 independently of its helicase activity (Weinstein and Rothstein, 2008). To determine the role of Sgs1 helicase activity in meiotic recombination, we analyzed cells expressing the helicase-dead *sgs1-K706A* mutation. Like the *sgs1*-Δ*N82*

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allele, *sgs1-K706A* mutation conferred very similar phenotypes to those conferred by P_{CLB2} alleles of the STR genes (Figure S4). Thus, Sgs1-Top3 interaction and Sgs1 helicase activity are required to suppress aberrant meiotic recombination.

The Catalytic Activity of Top3 is Required to Suppress Aberrant Recombination

The data above imply that Sgs1 helicase works in concert with the strand-passage activity of Top3-Rmi1 for all of its roles in meiotic JM processing. However, a non-catalytic function for Top3-Rmi1 could be envisioned, such as targeting Sgs1 to JMs. Precedent for a stimulatory function of Top3, independent of its decatenase activity, is seen for the DSB resection function of the STR complex (Cejka et al., 2010a; Niu et al., 2010).

We tested whether the strand-passage activity of Top3 is required for meiotic JM processing by mutating the active-site tyrosine (Y356F). Because *top3-Y356F* cells share the slow growth and genome instability phenotypes of the *top3*Δ null, these strains were made heterozygous with P_{CLB2}-*TOP3*. Physical assays showed that the recombination phenotypes of *top3-Y356F* cells were very similar to those of P_{CLB2}-*TOP3* cells (Figure 4A–D; Figure S5). This included near normal levels of crossovers and noncrossovers, elevated ectopic crossovers (Figure 4A,B), synthetic interaction with mutations in the resolving endonucleases (Figure 4C,D), and altered timing of crossovers relative to noncrossovers (Figure S5).

The top3-Y356F Allele Causes Recombination-Dependent Meiotic Catastrophe

Although P_{CLB2}-*TOP3* and *top3-Y356F* mutants confer indistinguishable recombination phenotypes, a stark difference was seen for chromosome segregation (Figure 4E,F). We showed previously that while *sgs1* mutants undergo meiotic divisions efficiently and form spores, more than half the cells had unsegregated DNA masses indicating defective chromosome segregation (Oh et al., 2008)(Figure 4E,F). A similar defect was seen in the current study for both P_{CLB2}-*TOP3* and P_{CLB2}-*RMI1* mutants (Figure 4E,F): both strains completed divisions efficiently, as scored by the appearance of two and then four DAPI-staining bodies, but additional unsegregated DNA masses were detected in a majority of cells. Quantification of this defect indicates that P_{CLB2}-*TOP3* mutation has a greater impact on chromosome segregation than either P_{CLB2}-*SGS1* or P_{CLB2}-*RMI1* (Figure 4F).

Strikingly, nuclear division completely failed in *top3-Y356F* cells, even though spore formation was efficient indicating progression of the meiotic program (Figure 4E,F). This block to chromosome segregation is identical to the “meiotic catastrophe” described for mutants defective for multiple JM resolving enzymes (De Muyt et al., 2012; Jessop and Lichten, 2008; Oh et al., 2008; Zakharyevich et al., 2012). To test whether meiotic catastrophe in *top3-Y356F* cells results from problems in meiotic S-phase or from defects in recombination we mutated *SPO11*, which encodes the transesterase responsible for programmed DSB formation. In *spo11*Δ null mutants, meiotic divisions occur efficiently even though the absence of recombination causes homologs to segregate randomly. Meiotic catastrophe in *top3-Y356F* cells was completely suppressed by *spo11*Δ mutation (Figure 4G,H) showing that the block to chromosome segregation is recombination dependent. Given that *sgs1* mutation suppresses the slow-growth defect of *top3* cells (Gangloff et al.,

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1994), we asked whether it could suppress the meiotic catastrophe of *top3-Y356F* cells. Although a small fraction (≤10%) of P_{CLB2}-*SGS1 top3-Y356F* cells appeared to complete one or both divisions, meiotic catastrophe was still observed in ≥90% of cells (Figure 4G,H).

A Subset of JMs Persists in top3-Y356F Cells

The high levels of recombinant products (both crossovers and non-crossovers; Figure 4A,B), detected in *top3-Y356F* mutants indicate that JM resolution must be relatively efficient. Thus, we assumed that chromosome segregation is blocked by a small but persistent subset of unresolvable JMs. Direct evidence that this is the case was obtained by 2D gel analysis of JMs from *top3-Y356F* cells (Figure 4I). Even at 13 hours after induction of meiosis, several hours after cells have formed spores, JMs were still detected in *top3-Y356F* cells (Figure 4J). In contrast, JMs were barely detectable in wild-type and P_{CLB2}-*TOP3* cells.

Top3-Rmi1 Acts Late in Meiotic Prophase to Promote Chromosome Separation

The block to chromosome separation seen in *top3-Y356F* cells could be caused solely by early defects in JM formation that create unresolvable structures. Arguing against this idea, the recombination defects of P_{CLB2}-*TOP3* and *top3-Y356F* cells are indistinguishable (Figure 4), yet P_{CLB2}-*TOP3* cells do not experience meiotic catastrophe. Alternatively, Top3 may be required both to regulate JM formation in early prophase, and to resolve a subset of JMs in late prophase. To explain the absence of meiotic catastrophe in the P_{CLB2}-*TOP3* mutant, we propose that these cells contain sufficient residual Top3 protein to perform the postulated late function and, as such, are able to resolve most of the structures that would otherwise block chromosome segregation.

To test these ideas, we constructed an auxin-inducible degron (AID) allele of *TOP3* (*AID-TOP3*) in a strain that also carries an estrogen-inducible allele of the *NDT80* gene (P_{GAL1}-*NDT80*; Figure 5; see Experimental Procedures)(Carlile and Amon, 2008). This system allowed Top3 to be degraded in pachytene-arrested cells (prior to *NDT80* expression), after JMs have been formed, but prior to their resolution (Figure 5A,B,C). Following induction of *NDT80* with estradiol, meiosis resumed and progression was monitored microscopically (Figure 5D,E).

AID-induced degradation of Top3 in late prophase completely blocked nuclear divisions, while the majority of control cells (without auxin) completed divisions within 2 hrs of Ndt80 expression (Figure 5E). Addition of auxin at different times allowed us to define a Top3 execution point relative to the induction of Ndt80 expression by estradiol (Figure 5E). Remarkably, addition of auxin 30 minutes after the addition of estradiol was still able to completely block chromosome separation. Similar data were obtained for an Rmi1-AID degron (Figure S6). Thus, chromosome separation specifically requires a late function of Top3-Rmi1 that is executed at the time of general JM resolution.

Late Action of Top3-Rmi1 Promotes a Subset of Recombinants and Suppresses Ectopic Recombination

To further understand the late function of Top3-Rmi1 in meiosis, we monitored progression of recombination at *HIS4::LEU2* in time-course experiments with and without AID-Top3

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degradation (Figure 6). Consistent with the Ndt80 dependence of JM resolution, ~85% of crossovers formed after estradiol addition. Previous analysis indicated that noncrossover formation is Ndt80 independent (Allers and Lichten, 2001). However, ~50% of noncrossovers at *HIS4::LEU2* also formed after addition of estradiol, suggesting the existence of a distinct class of Ndt80-dependent noncrossovers (Figure 6A,B).

When Top3 was inactivated, appearance of all recombinants was delayed (by ~18–35 minutes), indicating a general influence of Top3 on JM resolution. In addition, a subset of recombinants required the late action of Top3. Specifically, crossovers were reduced by ~16% in auxin-treated cells, while noncrossovers were reduced by ~26%. Thus, around half of the Ndt80-dependent noncrossovers are Top3 dependent.

In contrast to allelic crossovers and noncrossovers, late inactivation of Top3 caused a 2.7-fold increase in ectopic crossovers (Figure 6A,B). By comparison, early Top3 inactivation caused a 6.5-fold increase (Figure 1E). These data imply that Top3 must act throughout meiotic prophase, during both JM formation and JM resolution, to efficiently limit ectopic recombination.

2D gel analysis showed that JM resolution was delayed and inefficient following late inactivation of AID-Top3 (Figure 6C,D). As seen in the *top3-Y356F* mutant, a subset of JMs remained unresolved at late times, both during and after meiotic divisions (which occur at ~8–9 hrs; Figure 6E) accounting for the block to chromosome separation. In general, persistent JM signals appeared blurrier and streakier suggesting greater structural heterogeneity (Figure 6C). Inefficient resolution was seen for all JM species, but multi-chromatid JMs showed by far the greatest defect (Figure 6D). Very similar data were obtained for the Rmi1-AID degron (Figure S7) implying that the single-strand decatenase function of the Top3-Rmi1 complex is essential for the late stages of meiotic recombination.

Discussion

Preference of the Top3 topoisomerase activity for decatenation versus relaxation is enhanced by Rmi1, which stabilizes the open-gated form of the enzyme following Top3-catalyzed nicking of single-stranded DNA (Cejka et al., 2012). Thus, Top3-Rmi1 defines a potent single-strand decatenase. Canonical models of meiotic recombination did not predict a pervasive role for such an activity (Allers and Lichten, 2001; Andersen and Sekelsky, 2010; Bishop and Zickler, 2004; Borner et al., 2004; Hunter and Kleckner, 2001). As such, our results compel significant revision of these models.

Early Roles of Top3-Rmi1 are Interdependent With Sgs1

We previously inferred that Sgs1 limits aberrant JMs by removing excess recombinational interactions resulting from promiscuous strand invasion by both DSB ends and with multiple template chromosomes (Oh et al., 2007). Theoretically, Sgs1 could act alone in this process by unwinding nascent strand-exchange intermediates. Indeed, D-loops can be unwound by the BLM helicase *in vitro* (Bachrati et al., 2006; van Brabant et al., 2000). However, our data indicate that DNA helicase and decatenase activities of the STR complex act in an interdependent fashion to suppress aberrant meiotic recombination (Figure 7A). In an

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independent study, Lichten and colleagues have reached the same conclusion (Kaur, De Mut and Lichten, personal communication).

Our analysis of *sgs1* alleles defective for Top3 interaction (*sgs1-ΔN82*) and helicase activity (*sgs1-hd*)(Figure S4), suggest a model for early JM processing in which Sgs1 both unwinds duplex DNA and targets Top3-Rmi1 to one of the resulting single strands. Requirement for Sgs1 helicase activity implies that processed intermediates do not contain preexisting ssDNA for Top3-Rmi1 decatenation.

Requirement for the compound activity of STR presumably reflects *in vivo* properties of nascent strand-exchange intermediates. One possibility is that these structures are topologically constrained by bound proteins (Figure 7B). In addition, strand-invasion intermediates may be topologically more complex than typically envisioned. Such complexity could include known substrates of Top3-Rmi1, such as hemicatenanes, but also entanglements caused by plectonemic wrapping of displaced single strands, discontinuous heteroduplexes, displaced ends, cryptic dHJs (prior to second-end capture), double D-loops and other multiple invasion products (Figure 7B). Such complexity is implied by our identification of both mcJMs (Oh et al., 2007) and recombinant JMs that contain crossover strands (rJMs)(Oh et al., 2008). The latter must derive from endonuclease-dependent cleavage of one or more HJs, but the involved duplexes remain connected by additional unknown structures.

Both bound proteins and topological complexity may render nascent JMs resistant to simple unwinding by Sgs1/BLM. Consonant with this possibility, *in vitro* analysis by Heyer and colleagues shows that while protein-free D-loops are dissociated by Sgs1 and BLM, D-loops formed by Rad51 are not. Strikingly, Rad51-made D-loops are dissociated by Top3-Rmi1 and TOPIIIα-RMI1/2 (W.D. Heyer, personal communication; see the accompanying manuscript).

An Essential Role for Top3-Rmi1 to Remove Recombination-Dependent Chromosome Entanglements

Suppression of the sporulation defect of the *top3*Δ null mutant by *spo11* mutation led Gangloff et al. (1999) to conclude Top3 is required to resolve recombination-dependent entanglements. Our data now provide direct evidence that a subset of JMs remains unresolved in both the *top3-kd* mutant and when Top3 or Rmi1 are depleted specifically in late prophase. However, even though unresolved JMs block chromosome separation, the meiotic program progresses and spores are formed. Notably, mature tetrad asci rarely form in *top3-kd* and *Top3-AID* mutants (as previously noted in mutants experiencing meiotic catastrophe), explaining the previous assumption that *top3* mutants fail to sporulate.

Top3 is Required Throughout Meiotic Prophase

With alleles that inactivate Top3-Rmi1 early in meiosis, it was impossible to discern whether the decatenase also acts at the resolution step of meiotic recombination. Using degron alleles, we have shown that late action of Top3-Rmi1 generally accelerates JM resolution, and is essential for timely removal a subset of structures that otherwise block

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chromosome segregation. Notably, we have never observed meiotic catastrophe for any *sgs1* allele, including the *sgs1*Δ null, *sgs1-K706A*, *sgs1-ΔN82* and an Sgs1-AID degron (Figure S4 and not shown). Thus, the essential role of Top3-Rmi1 in chromosome segregation appears to be independent of Sgs1 thereby defining a temporally and functionally distinct role for the decatenase in meiosis. Two possibilities could explain why Sgs1 is not essential for chromosome segregation: (i) pertinent structures already contain ssDNA that can be targeted for decatenation by Top3-Rmi1; or (ii) alternative processes provide ssDNA for Top3-Rmi1 decatenation. The latter could include other helicases, excision tracks from DNA mismatch repair, and forces generated by bulk chromosome compaction and separation.

A Subset of Recombinants Requires Late Action of Top3-Rmi1

Our analysis points to three classes of noncrossover that differ with respect to their dependence on Ndt80 and Top3-Rmi1 (Figure 6B). Ndt80 promotes expression of over 200 meiotic genes to promote late prophase events that commit cells to meiotic divisions (Winter, 2012). These include JM resolution, which is controlled by Ndt80-dependent expression of polo-like kinase Cdc5/Plk1 (Sourirajan and Lichten, 2008). Allers and Lichten (2001) showed that most noncrossovers form independently of Ndt80/Cdc5. Moreover, Ndt80/Cdc5-independent noncrossovers require STR and, in its absence, noncrossovers become dependent on both Ndt80/Cdc5 and the structure selective endonucleases (Figure 3) (De Muyt et al., 2012; Zakharyevich et al., 2012). These observations support a model in which the fate of meiotic recombination events is decided early, with noncrossovers forming via synthesis-dependent strand annealing and only crossovers involving formation of metastable intermediates such as dHJs (Bishop and Zickler, 2004).

This binary model is questioned by our observation that around half the noncrossovers at *HIS4::LEU2* form after Ndt80 expression, and half of these require late action of Top3-Rmi1 (Figure 6). Top3-Rmi1 dependency is compatible with a class of noncrossovers arising from Cdc5-dependent dHJ dissolution, as opposed to Cdc5-independent SDSA. Consistently, genome-wide analysis of heteroduplex from hybrid yeast strains has shown that up to 35% of noncrossovers contain the trans-heteroduplex signature of dHJ dissolution (Martini et al., 2011). However, trans-heteroduplex could also derive from independent SDSA events by the two DSB ends. In this scenario, the ensuing JMs must remain trapped in a way that their resolution necessitates both Cdc5 and Top3-Rmi1. Analysis of ectopic recombination further supports the existence of a Top3-Rmi1 dependent dHJ-dissolution pathway of noncrossover formation that protects the genome from gross chromosomal rearrangements.

Experimental Procedures

Yeast Strains and Tetrad Analysis

Full genotypes are shown in Table S2. The *HIS4::LEU2* locus has been described (Hunter and Kleckner, 2001). Tetrad analysis was performed using standard techniques as described in Oh et al. (2007). Map distances and NPD ratios were calculated using Stahl Lab Online Tools (http://molbio.uoregon.edu/~fstahl/). *top3-Y356F* was created using QuikChange II

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Site-Directed Mutagenesis Kit (Agilent Technologies Inc.). The promoters of *TOP3* and *RMI1* were replaced with the *CLB2* promoter using the pFA6a-*KanMX6-pCLB2-3HA* cassette (Lee and Amon, 2003). The AID system (Nishimura et al., 2009) was optimized for meiosis by replacing the promoter of P_{ADH1}-*OsTIR1* with the *CUP1* promoter. Top3 and Rmi1 fusions to a minimal AID degron were constructed using plasmid p7aid-9m as template for PCR-mediated allele replacement (Morawska and Ulrich, 2013). Estrogen-inducible *IN-NDT80 GAL4-ER* has been described (Benjamin et al., 2003; Carlile and Amon, 2008; Picard, 1994).

Meiotic Time Courses and DNA Physical Assays

Detailed protocols for meiotic time courses and DNA physical assays have been described (Oh et al., 2009). Error bars show averages (±SEM) from three to six experiments.

Light Microscopy

To analyze of meiotic divisions and sporulation, cells were fixed in 40% ethanol 0.1 M sorbitol, stained with DAPI, and ~200 cells were categorized for each time point. DAPI-stained cells were mounted in antifade (Prolong, Invitrogen) and images captured using a Zeiss AxioPlan II microscope, Hamamatsu ORCA-ER CCD camera and Volocity software.

Western Blot Analysis

Whole cell extracts were prepared using a TCA extraction method, essentially as described (Johnson and Blobel, 1999). Following SDS-PAGE and Western analysis, an anti-c-Myc monoclonal antibody (Roche; 11667149001) was used to detect Top3-AID-9myc and Rmi1-AID-9Myc.

Auxin-Induced Degradation of Top3 and Rmi1

5 hrs after induction of meiosis, $CuSO_4$ (50 μM) was added to induce expression of P_{CUP1}-*OsTIR1*. Cell cultures were split and 2 mM auxin (3-indoleacetic acid, Sigma I1375-0, dissolved in DMSO) or DMSO alone was added at indicated times (Figures 5 and 6). At 7 hrs, 1 μM estradiol (Sigma E8875 in DMSO) was added to induce *IN-NDT80*. Cell samples were processed to monitor Top3-AID-9Myc or Rmi1-AID-9Myc protein levels, meiotic divisions and recombination intermediates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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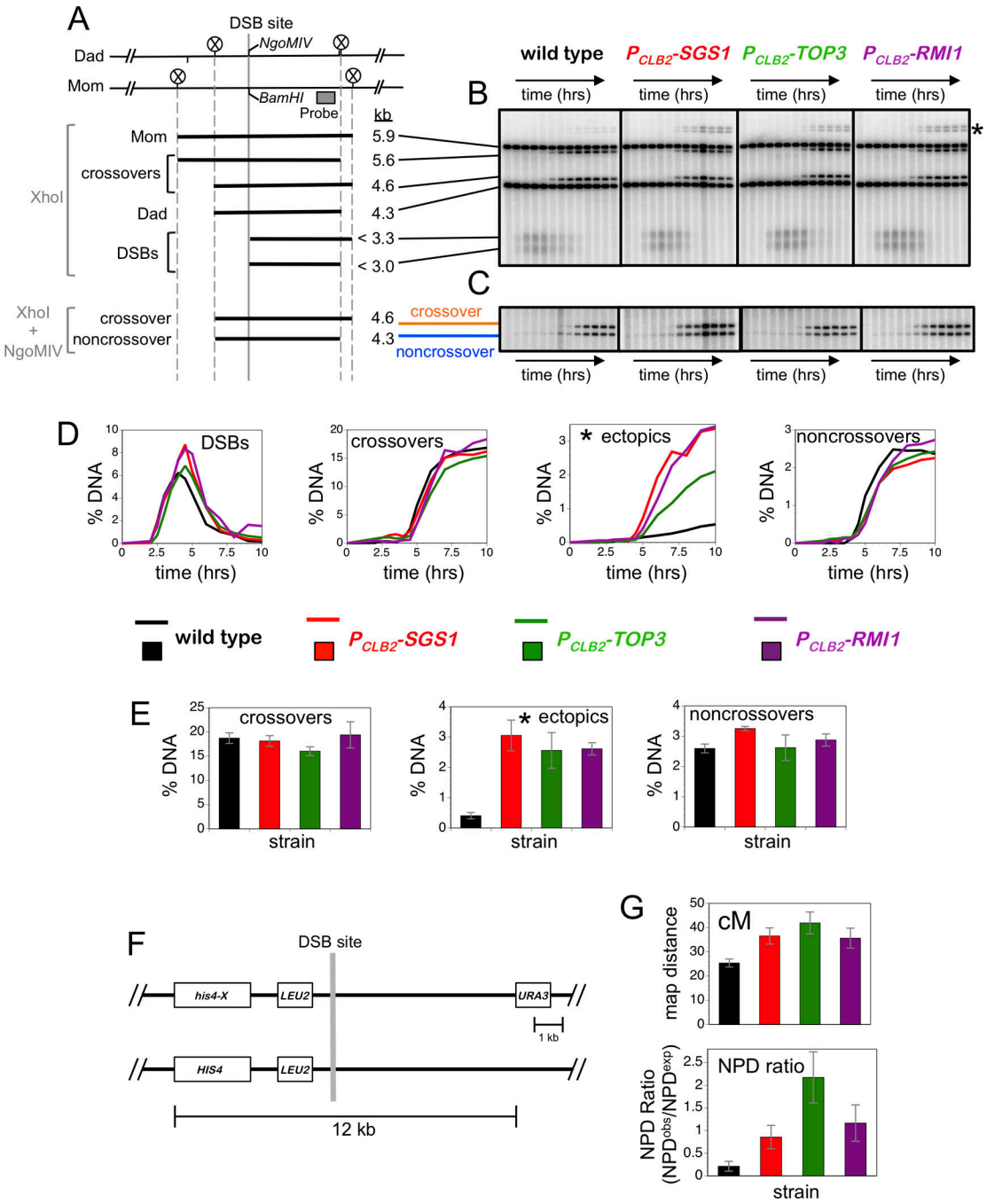

Figure 1. All STR Components are Required to Suppress Aberrant Recombination

(A) Map of the *HIS4::LEU2* hotspot highlighting the DSB site, diagnostic restriction sites and position of the probe used in Southern analysis. Sizes of diagnostic fragments are shown below. Circled Xs indicate XhoI sites.

(B) 1D Southern analysis of XhoI digested genomic DNA to monitor DSBs, allelic crossovers and ectopic crossovers (indicated by an asterisk). Time points are 0, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, and 13 hours.

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(C) 1D Southern analysis of XhoI+NgoMIV doubly digested genomic DNA to monitor noncrossover formation. Samples are from the same time courses shown in panel B.
(D) Quantitation of images shown in panels B and C. % DNA is percentage of total hybridizing DNA signal.
(E) Quantification of allelic crossovers, ectopic crossovers and noncrossovers after 13 hrs from multiple independent time-courses.
(F) Map of *HIS4::LEU2* hotspot showing flanking genetic markers.
(G) Map distances (upper panel) and NPD ratios (lower panel) for the interval shown in F. Data with error bars represent mean ± SEM. See also Figure S1 and Table S1.

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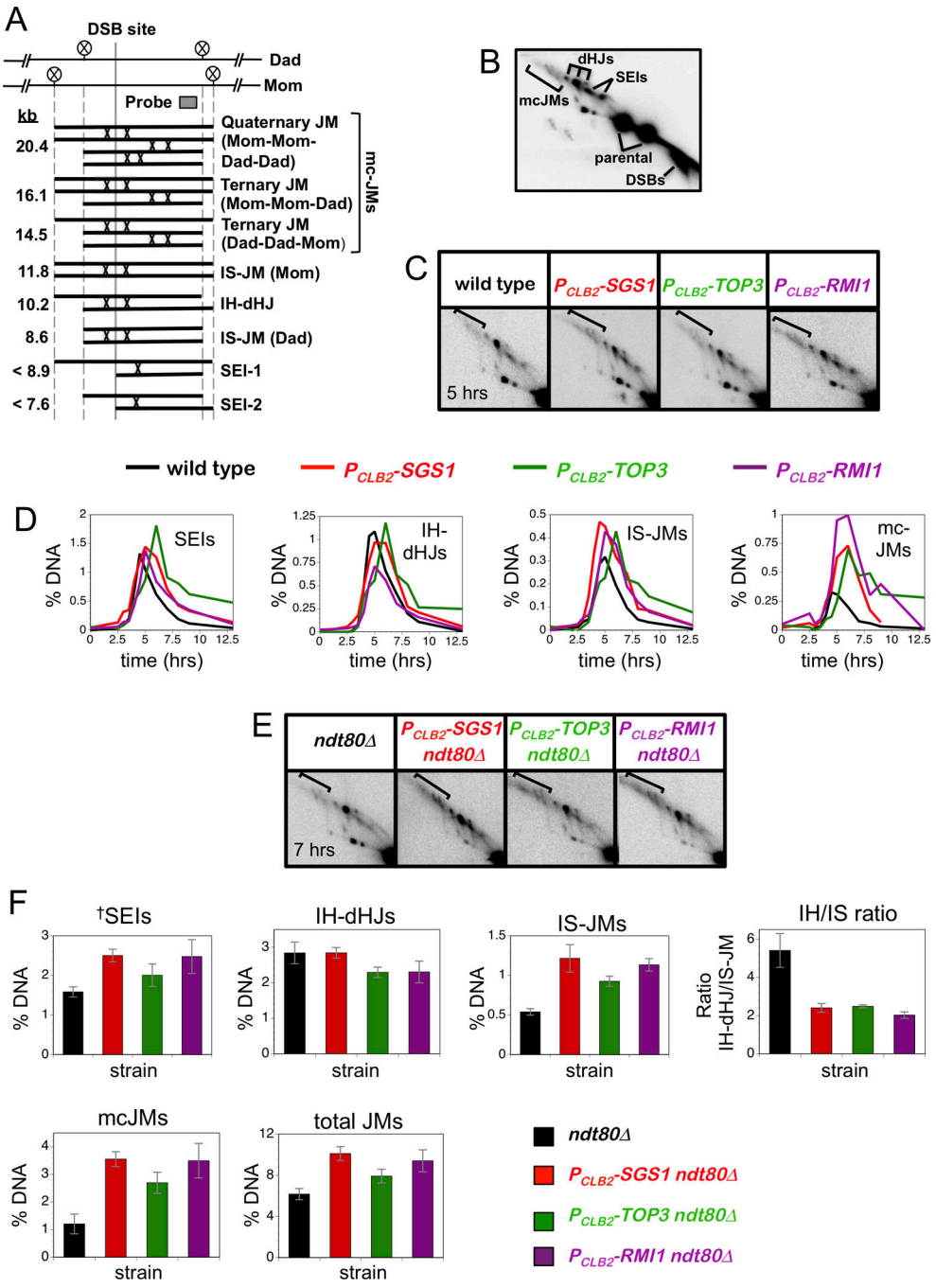

Figure 2. Altered Joint Molecule Spectra in P_{CLB2}-*TOP3* and P_{CLB2}-*RMI1* Mutants

(A) JM structures detected at *HIS4::LEU2*, from lowest to highest mobility. Positions of diagnostic XhoI sites (circled Xs) and the Southern probe are shown. IS-JM, inter-sister joint molecule; IH-dHJ, inter-homolog double Holiday junction; SEI, single end invasion; mc-JMs, 3-and 4-chromatid joint molecules.

(B) Southern blot of native/native 2D gel showing JMs detailed in panel A.

(C) 2D Southern analysis of JMs in wild type, P_{CLB2}-*SGS1*, P_{CLB2}-*TOP3 and* P_{CLB2}-*RMI1* strains.

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(D) Quantification of individual JM species. Percent DNA is percent of total hybridization signal.

(E) 2D Southern analysis of JMs in *ndt80*Δ, P_{CLB2}-*SGS1 ndt80*Δ, P_{CLB2}-*TOP3 ndt80*Δ *and* P_{CLB2}-*RMI1 ndt80*Δ strains.

(F) Quantification of individual JM species in *ndt80*Δ strains, total JMs and the ratio of IH-dHJs/IS-JMs. Percent DNA is percent of total hybridization signal.

Data with error bars represent mean ± SEM.

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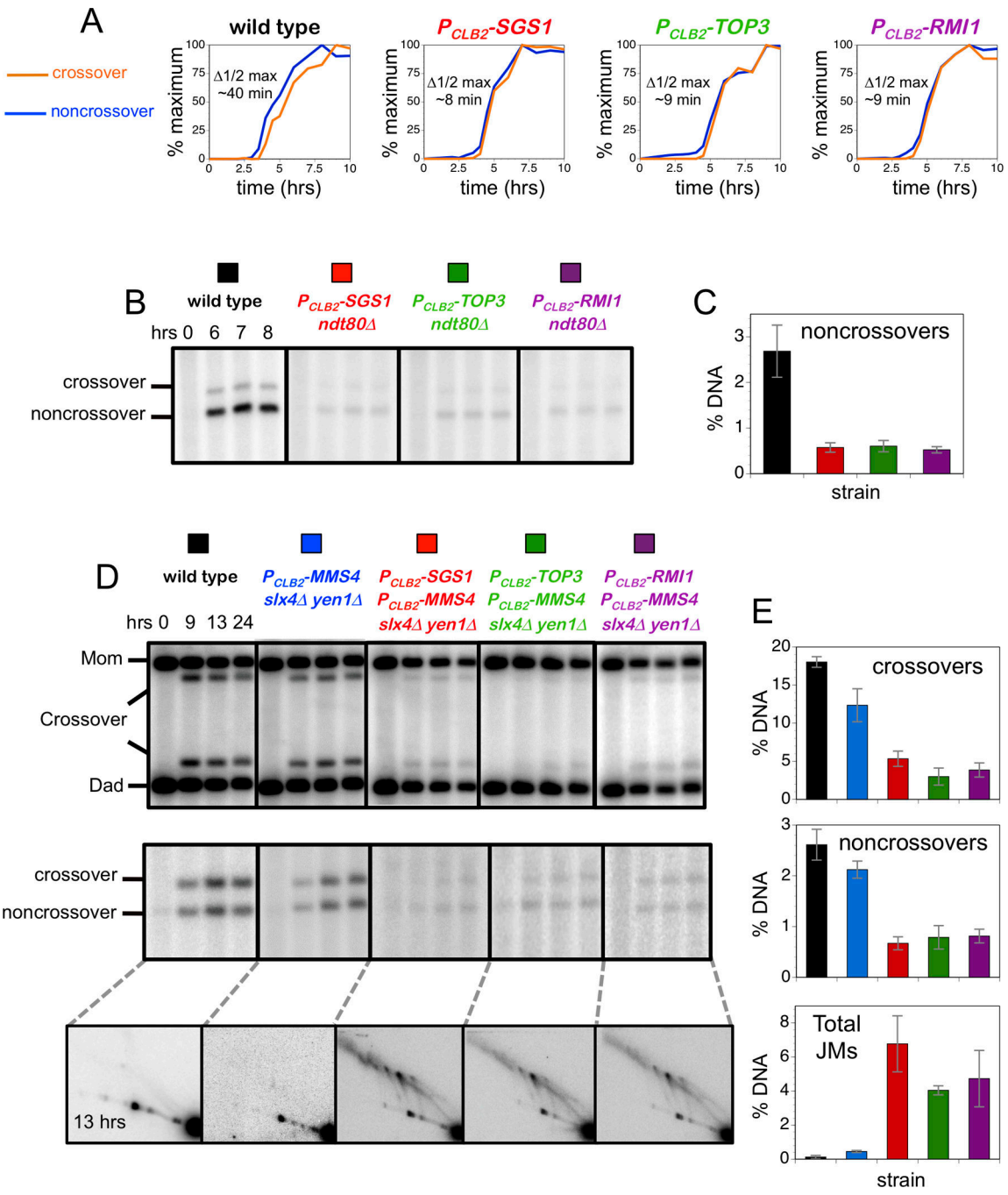

Figure 3. Crossovers and Noncrossovers in the P_{CLB2}-*TOP3* and P_{CLB2}-*RMI1* Mutants Require the Structure-Selective Endonucleases

(A) Normalized curves to compare the timing of crossovers and noncrossovers from the time-courses analysis shown in Figure 1C. Δ1/2 max is the difference between the times of the half maximum values.

(B) Representative Southern images of noncrossover analysis in the indicated *ndt80*Δ strains.

(C) Quantification of noncrossovers in *ndt80*Δ strains at 7 hrs.

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(D) Representative Southern images of crossover (upper panel), noncrossover (middle panel) and JM analysis (bottom panel) at indicated times.
(E) Quantification of crossovers, noncrossovers and total JMs at 13 hrs.
Data with error bars represent mean ± SEM. See also Figures S2, S3 and S4.

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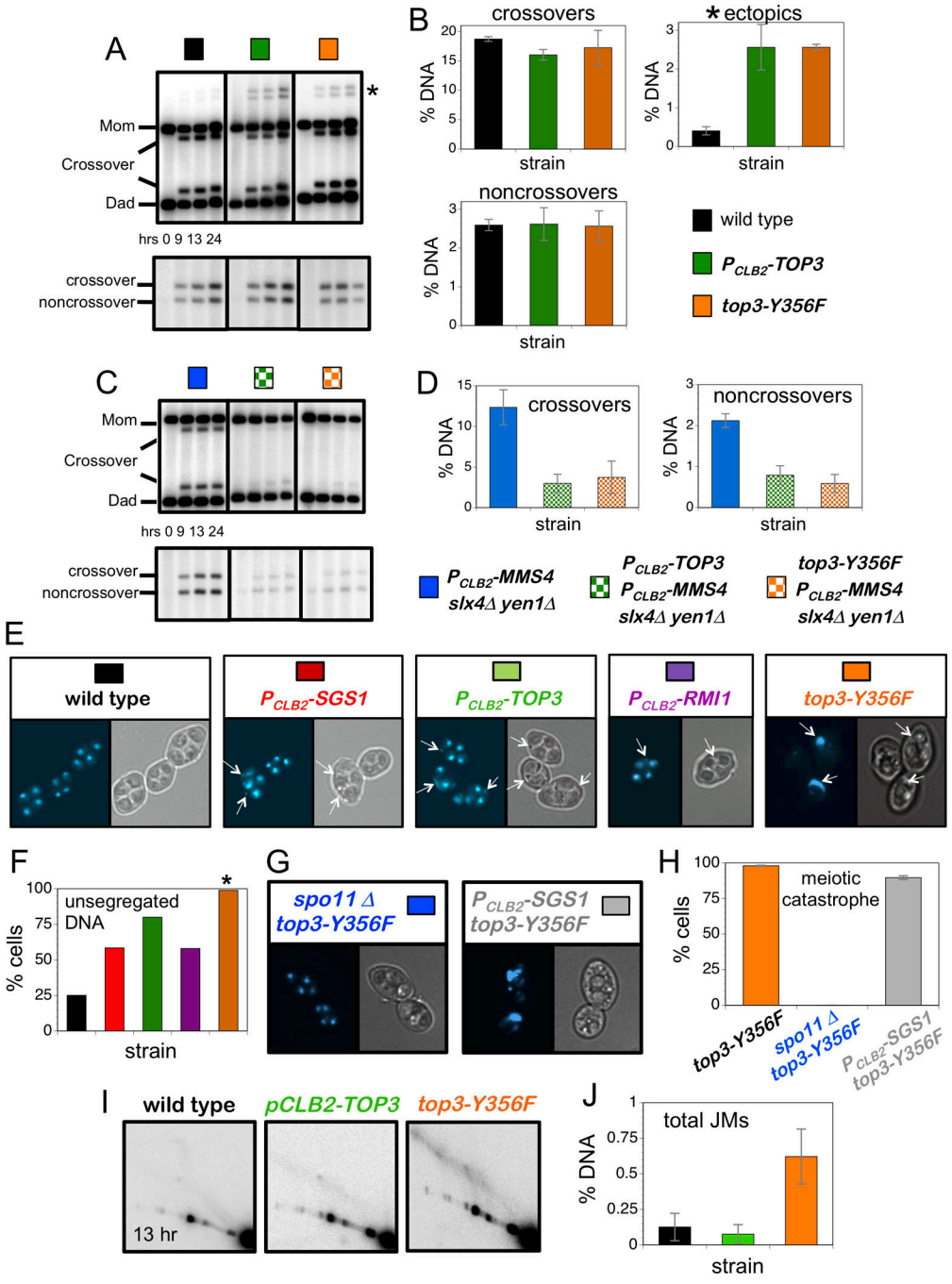

Figure 4. Single-Strand Decatenase Activity of Top3 Suppresses Aberrant Recombination and is Essential for Chromosome Segregation

(A) Representative Southern images of crossover (upper panel) and noncrossover (lower panel) analysis in indicated strains. Asterisk indicates ectopic crossover bands.

(B) Quantification of crossovers, ectopic recombination and noncrossovers at 13hrs.

(C) Representative Southern images of crossover (upper panel) and noncrossover (lower panel) analysis in indicated strains.

(D) Quantification of crossovers and noncrossovers at 13hrs.

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(E) Representative images of cells from indicated strains at 13 hrs. DAPI-stained and brightfield images of the same cells are shown. Arrows indicate unsegregated DNA masses.
(F) Quantification of cells containing unsegregated DNA masses. Colors correspond to the strains shown in panel E.
(G) Representative cells from *spo11*Δ *top3-Y356F* and P_{CLB2}-*SGS1 top3-Y356F* strain at 13 hrs.
(H) Quantification of cells showing meiotic catastrophe in *top3-Y356F*, *spo11*Δ *top3-Y356F* and P_{CLB2}-*SGS1 top3-Y356F* strains.
(I) Representative Southern images of 2D JM analysis at 13hrs.
(J) Quantification of total JM levels at 13 hrs in the strains shown in panel I.
Data with error bars represent mean ± SEM. See also Figure S4.

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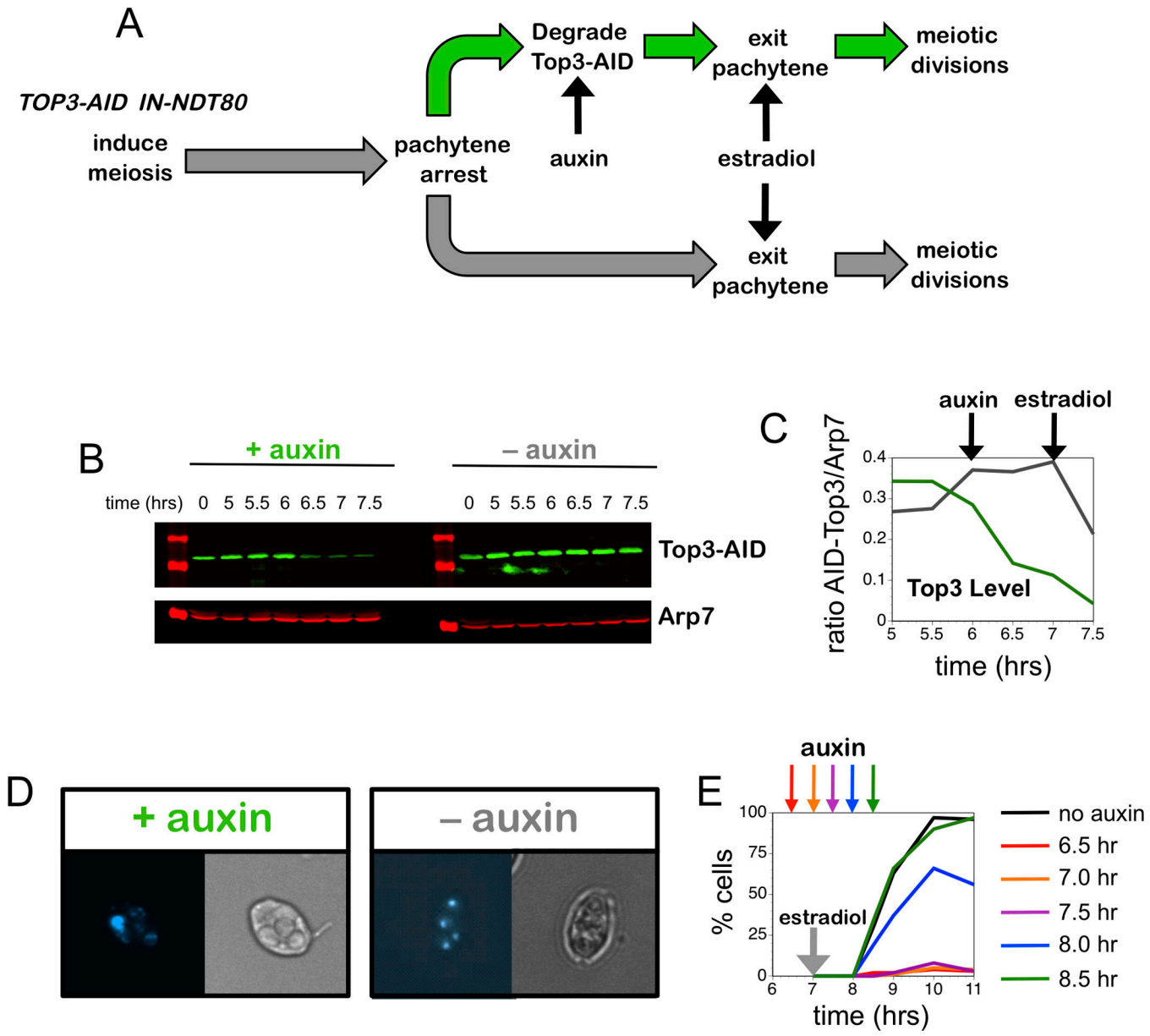

Figure 5. Top3 Acts Late in Meiotic Prophase to Promote Chromosome Separation

(A) Regimen to inactivate Top3-AID during late stages of meiotic prophase.

(B) Western images showing Top3-AID levels in cells with and without addition of auxin at 6 hrs. Arp7 was used as loading control.

(C) Quantification of Top3 levels from the experiment shown in panel B.

(D) Representative images of cells from subcultures with and without addition of auxin at 6 hrs and addition of estradiol at 7 hrs, sampled at 11 hrs. DAPI-stained and brightfield images of the same cells are shown.

(E) Quantification of nuclear divisions in subcultures with and without auxin treatment at indicated times, following induction of Ndt80 expression at 7 hrs.

See also Figure S6.

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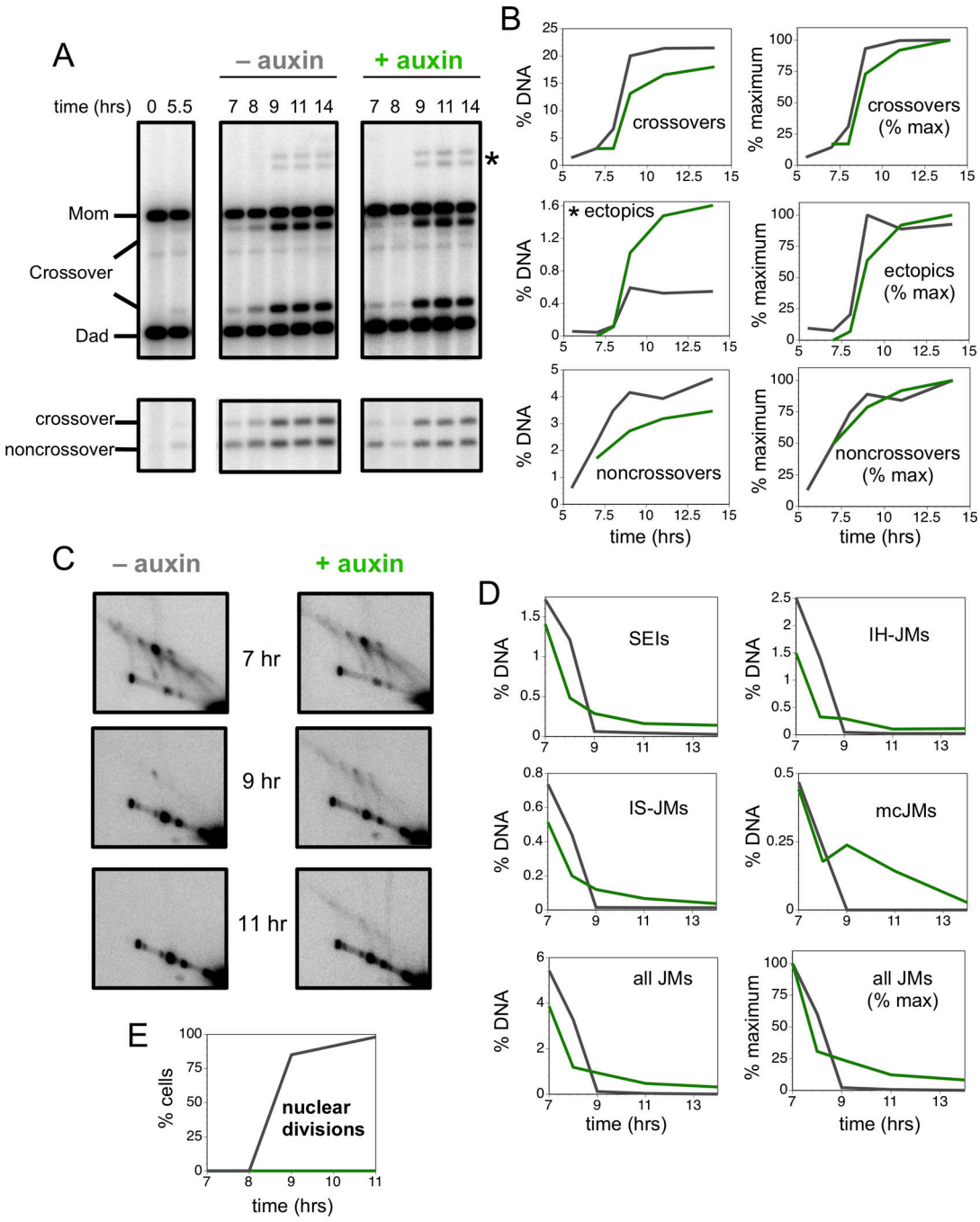

Figure 6. Late Action of Top3 is Required for Efficient Recombination and Suppression of Ectopic Crossovers

(A) Southern images of crossover (upper panels) and noncrossover (lower panels) analysis from cell subcultures with and without auxin treatment (Top3-AID degradation) at 6.5 hrs and induction of Ndt80 expression at 7 hrs.

(B) Quantification of allelic and ectopic crossovers, and noncrossovers from the experiment shown in panel A. Left panels show recombinants as percentage of total hybridizing DNA signal. Right panels show normalized curves to compare the timing of recombinants.

(C) Southern blot images of 2D JM analysis.

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(D) Quantification of individual JM species, total JM levels and normalized JM levels in cells with and without auxin treatment.
(E) Quantification of nuclear divisions in the two subcultures.
See also Figure S7.

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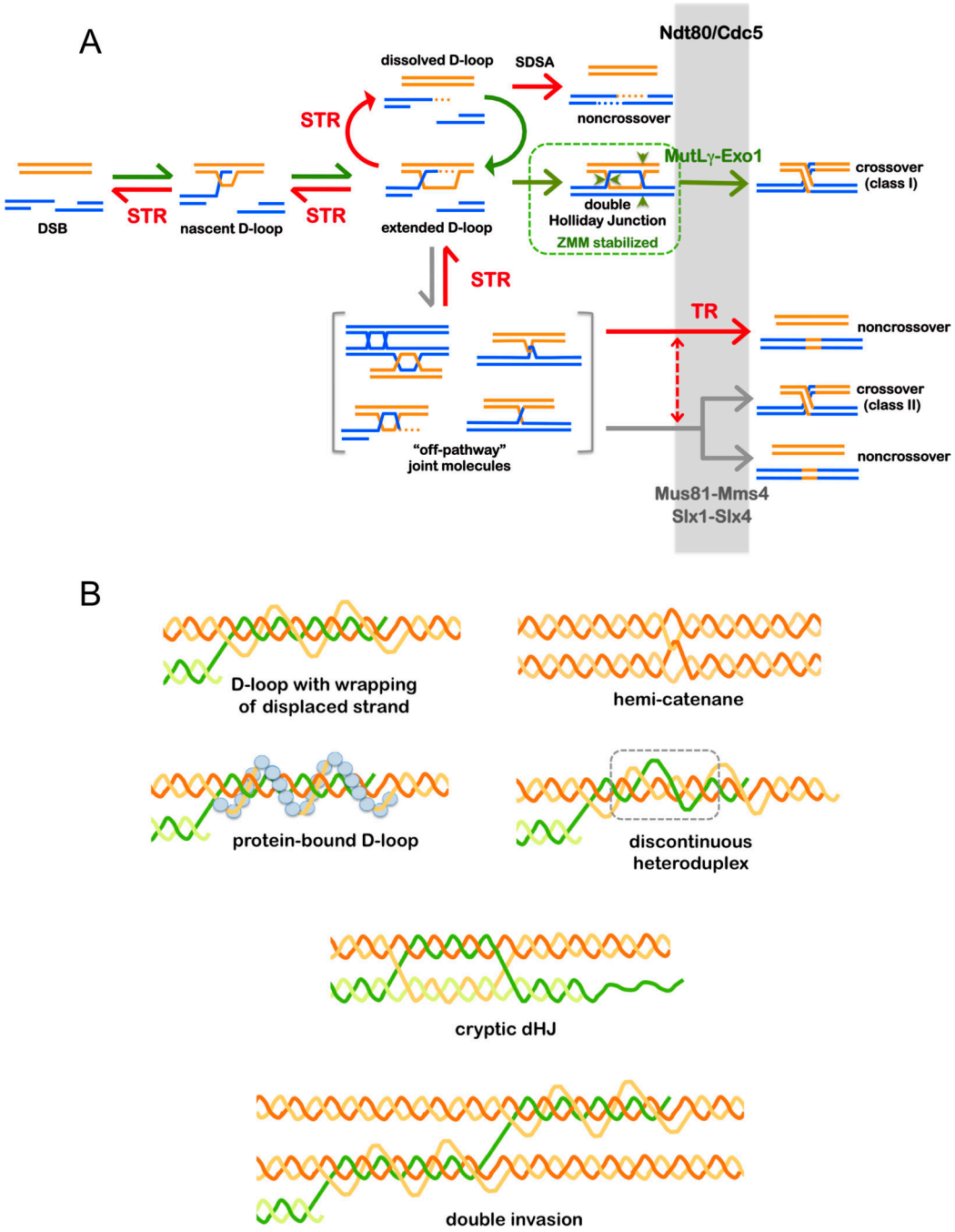

Figure 7. Roles of the Top3-Rmi1 Single-Strand Decatenase in Meiosis

(A) Pathways of meiotic recombination highlighting the prevalent role of STR's compound helicase-decatenase activity in regulating JM formation; and the late role of the TR decatenase in removing recombination-dependent entanglements. STR dissolves nascent and extended D-loops to reset DSBs. This process will reverse unproductive and excessive strand exchanges allowing DSBs to either engage in new rounds of strand invasion, or to anneal. Annealing can occur either as part of the SDSA process to form Ndt80/Cdc5-independent noncrossovers, or during second-end capture to form dHJ crossover precursors

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(Lao et al., 2008). The latter involves stabilization by ZMM proteins, such as MutSγ (Msh4-Msh5), which protect crossover-designated dHJs from STR-mediated dissolution. Crossover-specific resolution requires Cdc5/Plk1 and the Exo1-MutLγ ensemble. By dissolving nascent JMs, STR limits accumulation of off-pathway JMs that include mcJMs, trapped and dead-end intermediates, topologically-entrapped JMs, and structures that require resolution by the structure-selective endonucleases. Resolution of such structures involves Cdc5/Plk1, which generally activates JM resolution and directly activates Mus81-Mms4 (Matos et al., 2011). By promoting disassembly of synaptonemal complexes, Cdc5/Plk1 may also expose trapped JMs to the TR decatenase and the structure-selective endonucleases. A subset of noncrossovers derive from Cdc5/Plk1-dependent dissolution by TR. Resolution of some off-pathway JMs may require collaboration of TR and the structure-selective endonucleases, as indicated by the dashed arrow. At late stages, the TR decatenase may act broadly to remove residual entanglements from all JMs.

(B) Potential topological constraints and complexities in meiotic JMs that might necessitate a general requirement for the Top3-Rmi1 single-strand decatenase.