Removal of Spo11 from meiotic DNA breaks *in vitro* but not *in vivo* by Tyrosyl DNA Phosphodiesterase 2

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ABSTRACT

Meiotic recombination events are initiated by DNA double-strand breaks (DSBs) created by the topoisomerase-like protein, Spo11. Similar to type-II topoisomerases, Spo11 becomes covalently linked to the 5′ ends generated on each side of the DSB. Whilst Spo11-oligos—the product of nucleolytic removal by Mre11—have been detected in a number of biological systems, the lifetime of the covalent Spo11-DSB precursor has not been systematically determined and may be subject to alternative processing reactions. Here we explore the activity of human Tyrosyl DNA Phosphodiesterase, TDP2, on Spo11-DSBs isolated from *S. cerevisiae* cells. We demonstrate that TDP2 can remove Spo11 from natural ssDNA-oligos, and dsDNA ends even when in the presence of excess competitor genomic DNA. Interestingly, TDP2-processed Spo11-DSBs are refractory to resection by Exo1, suggesting that ssDNA generated by Mre11 may be essential *in vivo* to facilitate resection-dependent HR at Spo11-DSBs even if TDP2 were active. Moreover, although TDP2 can remove Spo11 peptides *in vitro*, TDP2 was unable to remove Spo11 *in vivo*—unlike during the repair of topoisomerase-induced DNA lesions. These results suggest that Spo11-DNA, but not topoisomerase-DNA cleavage complexes, are inaccessible to the TDP2 enzyme, perhaps due to occlusion by higher order protein complexes resident at sites of meiotic recombination.

32 **INTRODUCTION**

33 In most organisms, meiotic recombination is essential to create genetically diverse, haploid
34 genomes suitable for sexual reproduction. In meiosis, homologous recombination (HR)
35 events are initiated by DNA double-strand breaks (DSBs) created by Spo11, a topoisomerase-
36 like enzyme most closely related to the catalytic 'A' subunit of the archael type-IIB
37 Topoisomerase VI (1–3). TopoVI functions as an A_2B_2 heterotetramer, and the recent
38 identification of the eukaryotic 'B' subunit, Top6BL, in mouse, plants and budding yeast
39 indicates that the active form of Spo11 is similarly heterotetrameric (4, 5). Like other type-II
40 and type-IIB topoisomerases, the Spo11 monomers become covalently attached via a
41 phosphotyrosine bond to the 5′ ends generated on each side of the DSB during cleavage (6–
42 8). In wildtype *S. cerevisiae* cells, such covalent Spo11-DSBs are transient, being rapidly
43 processed by the nuclease activity of the MRX/N complex (Mre11, Rad50 and Xrs2/Nbs1),
44 which is stimulated by Sae2, the yeast orthologue of human CtIP (9–14). This coordinated
45 reaction generates a covalent single-stranded Spo11-oligonucleotide complex, flanked by a
46 nick or short ssDNA gap on the 5′-ending strand that enables the DSB to be subsequently
47 channelled into homologous recombination repair via the onset of long-range ssDNA
48 resection catalysed by Exo1 (9, 15–17). Spo11-oligo complexes are formed in an
49 evolutionarily divergent yeast, *Schizosaccharomyces pombe* (18), and are also detectable in
50 mouse spermatocytes (10, 19), suggesting that nucleolytic release is the default mode of
51 Spo11 removal in both unicellular and multicellular eukaryotes.

52

53 However, whilst the product of this nucleolytic reaction—Spo11-oligos—have been detected
54 in numerous biological systems, the lifetime of the covalent DSB precursor has not been
55 systematically determined and may be subject to alternative processing reactions. For
56 example, in *S. pombe*—and unlike in *S. cerevisiae* (2)—covalent Spo11-DSBs are readily
57 detected in wildtype cells (20), indicating that DSB formation and the initiation of repair can
58 be temporally separated. Moreover, inactivation of the canonical Spo11 release reaction in *C.*
59 *elegans* (via mutation of COM-1, the CtIP/Sae2 orthologue) results in repair of DSBs by the
60 non-homologous endjoining (NHEJ) pathway and aberrant chromosome segregation (21, 22).
61 These observations suggest that delayed onset of meiotic DSB resection may be especially
62 problematic for meiotic cells, and further suggest there must be a COM-1-independent

63 mechanism to remove Spo11 in *C. elegans*, such that the DSBs can be ligated using the end-
64 joining apparatus.

65

66 In vegetative cells, the usually benign and transient protein-linked DNA breaks created by
67 Topoisomerase I (Top1) and II (Top2) as part of their reversible catalytic cycle may become
68 a stable and toxic covalent-DNA intermediates (23). This conversion is exploited in
69 chemotherapy by the use of drugs such as camptothecin and etoposide, which stabilise the
70 covalent cleavage-complex intermediate (Top1cc and Top2cc respectively), thereby
71 sensitising cells that have a relatively greater reliance on topoisomerase activity compared to
72 other tissue. Presumably because such topoisomerase failures are relatively common even in
73 unchallenged cells, two classes of enzymes exist that are capable of handling such lesions:
74 Tyrosine phosphodiesterase 1 (Tdp1), which primarily resolves the 3′ covalent
75 phosphotyrosine links created by Top1 (24), and tyrosine phosphodiesterase 2 (TDP2, also
76 named TTRAP), which primarily resolves 5′ phosphotyrosine links created by Top2 (25).
77 Some enzymatic overlap between the activities and substrates of Tdp1 and TDP2 exists,
78 suggesting some functional redundancy *in vivo* (25, 26). Nevertheless, the primary activity of
79 Tdp1 activity aids the conversion of Top1 lesions into ligatable single-strand breaks (SSBs),
80 whereas TDP2 converts Top2 lesions into DSB ends suitable for direct repair by the NHEJ
81 pathway. The reaction product created by TDP2 contrasts with the 3′ single-stranded ends
82 generated as a result of MRX/N activity, and which are suitable for HR (9, 15, 27).
83 Interestingly, TDP2 is expressed in mouse and human testes tissue (28), leading to the
84 possibility that TDP2 may be able to process a subset of Spo11-DSB intermediates.

85

86 Here we use ectopic expression and *in vitro* biochemistry to explore the activity of human
87 TDP2 on Spo11-DSBs generated in *S. cerevisiae* meiotic cells. Collectively, our results
88 indicate that while TDP2 is catalytically capable of removing Spo11 from both Spo11-oligos
89 and Spo11-DSB ends *in vitro*, TDP2 is unable to do so *in vivo*. This is despite TDP2
90 functioning efficiently to suppress the sensitivity of *mre11* or *sae2* mutants to topoisomerase
91 poisons. These results suggest that Spo11-DSBs, but not Topoisomerase-DSBs, are
92 inaccessible to the TDP2 active site, perhaps due to occlusion by higher order protein
93 complexes resident at sites of meiotic recombination. Moreover, we find that the 2 nt 5′
94 extensions revealed when Spo11 is removed from DSB ends by TDP2 *in vitro* are refractory

95 to nucleolytic resection by Exo1, suggesting that even were TDP2 to act during meiotic DSB
96 repair, it would nonetheless lead to DSBs being channelled away from the essential process
97 of homologous recombination. Overall, our findings provide insight into the mechanisms
98 involved in the resection and repair of protein-linked DSB ends created by topoisomerase-
99 like enzymes such as Spo11, and how they may differ to the repair of 'clean'-ended DNA
100 breaks created by site-specific nucleases.
101

102 **RESULTS**

103 **TDP2 removes Spo11 protein and peptides from single-stranded Spo11-oligo complexes.**
104 Meiotic recombination is initiated by DSBs created by the evolutionarily conserved Spo11
105 protein. As part of the catalytic reaction, Spo11 becomes covalently attached to the 5′ DSB
106 ends via a phosphotyrosyl bond. In *M. musculus*, *S. cerevisiae*, and *S. pombe*, Spo11 is
107 released from the DSB ends by the action of the evolutionarily conserved MRX/N nuclease
108 complex in conjunction with Sae2/CtIP/Ctp1, generating covalent Spo11-oligo complexes (9,
109 10, 18, 19, 29). However, whether alternative mechanisms of Spo11 end-processing are
110 possible has not been investigated.
111

112 To investigate whether Spo11-DSBs may also be a substrate for processing by TDP2—as are
113 Top2-induced DSBs (25)—we took advantage of the *S. cerevisiae* meiotic system in which
114 synchronised populations of cells undergo Spo11-DSB formation at hundreds of sites spread
115 across the genome (termed Spo11 hotspots), but in which TDP2 is not naturally expressed.
116

117 We first tested for the ability of recombinant TDP2 to remove Spo11 covalently attached to
118 ssDNA by enriching for the natural covalent Spo11-oligo complexes created by MRX and
119 Sae2-dependent processing of meiotic Spo11-DSBs in wildtype cells (Fig 1a). Cells in mid
120 meiotic prophase (4 hours after meiotic entry) were harvested, lysed, and Spo11-oligo
121 complexes isolated by immunoprecipitation using antibodies raised against a C-terminal
122 triple FLAG epitope (30).
123

124 Immunoprecipitates were washed and then the ssDNA component labelled at the terminal 3′
125 hydroxyl group using terminal deoxynucleotide transferase (TdT) and a radiolabelled
126 nucleotide (dCTP). Under the conditions employed, this reaction results in 1-4 radiolabelled
127 nucleotides being added to the 3′ OH end of the Spo11-oligo complex (D. Johnson and M.

128 Neale unpub. obs), which can be resolved via SDS-PAGE (Fig 1b). Recombinant human
129 TDP2 (25) was reacted with these "full-length" labelled Spo11-oligo complexes, or with
130 complexes pre-treated with either trypsin or proteinase K (Fig 1c-f). Based on substrate
131 specificity, trypsin is expected to leave twelve amino acid residues covalently attached to the
132 oligo complex via the phosphotyrosine bond, whereas proteinase K treatment will degrade all
133 but three amino acids in addition to the phosphotyrosine.
134
135 Following incubation with increasing concentrations of TDP2, reactions were halted by
136 adding formamide gel-loading buffer, then resolved on a 15% polyacrylamide gel under
137 DNA-denaturing conditions (Fig 1d-f). Under these conditions, untreated Spo11-oligo
138 complexes fail to enter the gel and are often lost from the wells during gel handling and
139 fixation (Fig 1d). By contrast, trypsin-treated molecules migrate as a heterogeneous smear,
140 presumably due to significant electrophoretic retardation caused by the remaining amino
141 acids bound to the DNA (Fig 1e). Proteinase K treated samples migrate more uniformly, as a
142 pair of broad peaks, consistent with the previously characterised dual size classes of Spo11-
143 oligo complex detected in *S. cerevisiae* (8-15 nt and 25-40 nt; Fig 1f; (9, 10)).
144
145 Consistent with experiments performed using synthetic phosphotyrosine ssDNA substrates
146 (25) or partially proteolysed Top2-DNA covalent complexes (31), we observed robust
147 activity of TDP2 against both the partially and fully proteolysed Spo11-oligo substrates (Fig
148 1e,f). In both cases, we observed the conversion of the radiolabelled signal to faster migrating
149 forms consistent with cleavage of the 5′ phosphotyrosine bond and complete removal of any
150 residual amino acids from the radiolabelled oligo.
151
152 Importantly, incubation of TDP2 with a radiolabelled oligononucleotide (i.e. without a
153 covalent protein attachment) resulted in no change in electrophoretic migration (Fig S1a),
154 confirming that the increased rate of migration observed above (Fig 1e,f) was not due to trace
155 nuclease activity within the TDP2 preparation, but instead was due to the expected cleavage
156 of the protein-DNA phosphotyrosyl bond.
157
158 Structural modelling of TDP2 indicates that the catalytic site is buried within the core of the
159 protein, which any phosphotyrosine substrate would be required to thread into to be cleaved
160 (32–34). Intriguingly, however, we also observed conversion of non-migrating to rapidly

161 migrating oligo complexes when TDP2 was reacted with full-length Spo11-oligos, albeit only
162 restricted to the reactions containing the higher enzyme concentrations (Fig 1d). These
163 observations support the view that TDP2 can remove both full-length and Spo11 protein
164 fragments from ssDNA ends, and that prior proteolytic degradation of the 5′-covalently
165 linked protein stimulates the enzymatic activity of TDP2, similar to what has been observed
166 for its activity on Top2-DNA intermediates (31).
167
168 **TDP2 removes Spo11 peptides from DSB ends.**
169 We next tested the ability of TDP2 to remove Spo11 from the naturally occurring double-
170 stranded DNA (dsDNA) ends generated during meiosis. As introduced above, in wildtype *S.*
171 *cerevisiae* cells, Spo11-DSBs are normally rapidly processed by MRX/Sae2 (liberating
172 Spo11-oligo complexes) and then subjected to Exo1-dependent resection to create long 3′
173 ending ssDNA tails. Thus, to enrich for a population of covalent Spo11-DSB molecules to
174 use as a substrate, and therefore to prevent such nucleolytic processing steps, we prepared
175 genomic DNA samples from *sae2*Δ cells in mid meiotic prophase (4 hours after meiotic
176 induction when DSBs are abundant). The resulting nucleic acid material is free from non-
177 covalently attached protein contaminants but, due to the *SAE2* gene deletion, retains covalent
178 Spo11-DSB intermediates (8, 35, 36). Samples were then incubated with and without TDP2
179 for up to 15 minutes, then subsequently reacted with lambda exonuclease—a 5′-3′
180 exonuclease with activity that depends on the presence of an unmodified 5′ phosphate group
181 on the substrate (37). Post-treatment with lambda exonuclease thus provides a sensitive test
182 for TDP2-dependent removal of the Spo11 moiety from the DSB end (Fig 2a).
183
184 To monitor the reactions, DNA was digested with *Pst*I, separated on an agarose gel,
185 transferred to nylon membrane and hybridised with a radiolabelled DNA probe that
186 recognises the *HIS4*::*LEU2* meiotic recombination hotspot (38). Preparation in this way
187 reveals three distinct bands, the upper most is the parental DNA molecule, and the lower
188 smaller bands are indicative of DSBs arising at the two strong hotspots present at this
189 genomic locus (Fig 2b). Treatment with lambda exonuclease alone resulted in no change in
190 the migration of the DSB bands—as expected due to the attached Spo11 protein preventing
191 5′-3′ resection (Fig 2b). By contrast, pretreatment with TDP2 led to complete disappearance
192 of the DSB bands—as expected if TDP2 was able to efficiently remove Spo11 from the DSB
193 end creating a clean 5′ phosphorylated substrate on which lambda exonuclease can act (Fig

194 2b). Notably, the parental DNA band was stable under these conditions indicating that the
195 exonuclease degradation was specific to the deprotected Spo11-DSB ends (Fig 2b). If we
196 assume there to be approximately 160 DSBs (39) within each replicated *S. cerevisiae* diploid
197 genome (~50 Mbp) there will be approximately one covalent Spo11-DSB end for every ~150
198 kb of genomic DNA. We conclude that recombinant TDP2 is able to efficiently remove
199 Spo11 from dsDNA ends even in the presence of a large excess of dsDNA.
200
201 **Exo1 is unable to resect Spo11-DSBs that have been processed by TDP2.**
202 As described above, the endogenous exonuclease involved in 5′-3′ resection of Spo11-DSBs
203 is Exo1 (9, 15, 17). We were thus interested to examine whether treatment of Spo11-DSBs
204 with TDP2 could enable resection by recombinant Exo1, perhaps indicating a potential
205 mechanism to bypass the requirement of MRX/N nuclease activity *in vivo*. To perform these
206 experiments, we isolated genomic DNA from *sae2*Δ cells as described above, incubated with
207 and without TDP2 for up to 15 minutes, then incubated with recombinant Exo1 rather than
208 lambda exonuclease. Unlike treatment with lambda exonuclease, Exo1 was unable to initiate
209 resection on Spo11-DSBs whether or not they had been processed by TDP2 (Fig 3a,b).
210
211 As a positive control to ensure that this inability to resect the Spo11-DSB end was not due to
212 a problem with our recombinant Exo1 preparation, we repeated the same set of assays with
213 genomic DNA isolated from an *exo1*Δ mutant (rather than *sae2*Δ). Under these conditions,
214 MRX and Sae2-mediated nucleolytic cleavage of Spo11 from the DSB *in vivo* results in a
215 short 3′ ssDNA tail (up to ~300 bp) (15). As expected, incubation of this substrate with
216 recombinant Exo1 resulted in specific degradation of the DSBs whether or not they were first
217 treated with TDP2 (Fig 3c,d).
218
219 As a final control, we confirmed that TDP2 was active in the Exo1 buffer by incubating the
220 Spo11-oligo substrate with TDP2 under the same conditions that the Exo1 resection assays
221 were performed (Fig S1b). Collectively these results demonstrate that whilst TDP2 may have
222 the necessary biochemical activity to remove covalently attached Spo11 from DSB ends, the
223 resulting clean DNA ends are a poor substrate for Exo1 to initiate resection.
224
225

226 **Exo1 is unable to resect Spo11-DSBs that have been blunted by Klenow fragment.**
227 Spo11 creates a 2 nt 5′ overhang at the DSB ends (7, 39, 40). Prior *in vitro* assays using
228 relatively short (~3.0 kb) plasmid fragments indicated that the preferred substrate for Exo1 is
229 a dsDNA end with a 3′ extension, similar in form to the resected DSBs created by
230 MRX/Sae2, and in agreement with our observations presented above (41). By contrast, Exo1
231 displayed reduced, but not abolished, activity on substrates with a 4 nt 5′ extension, and only
232 moderately reduced activity on a blunt ended substrate (41). These latter findings contrast
233 somewhat with our observations that even just a 2 nt 5′ overhang completely prevents Exo1-
234 catalysed resection of the TDP2-processed Spo11-DSBs (Fig 3a,b). A possible explanation
235 for the difference may be the ratio of dsDNA to DSB ends, which is ~100-fold greater in our
236 assay system—and a much closer mimic of the conditions present *in vivo* during meiosis.
237
238 To determine whether Exo1 was able to resect blunt DSB ends under these same '*in vivo*-
239 like' conditions, we repeated the experiments utilising the TDP2-processed *sae2*Δ genomic
240 DNA, but this time including a preincubation step with Klenow fragment and dNTPs to blunt
241 the 2 nt 5′ overhang prior to incubation with Exo1 (Fig 3g,h). Unlike for the partially resected
242 substrates, no resection by Exo1 was detectable even when DSB ends were first blunted by
243 Klenow fragment (Fig 3g,h). Taken together our results suggest that Exo1 is acutely sensitive
244 to the structure of the DNA end, with partially resected ends—such as those created by
245 MRX/Sae2-dependent processing—being a significantly favoured substrate when excess
246 competitor dsDNA substrate is present. We suggest that our observations may represent a
247 closer match to the *in vivo* situation, where—even in meiosis—DSB ends are relatively
248 infrequent compared to the intact double-stranded genomic DNA.
249
250 **Ectopic expression of TDP2 is unable to cleave Spo11 from DSB ends in *S. cerevisiae.***
251 An orthologue of the TDP2 protein is not present in *S. cerevisiae*. However, human TDP2
252 was identified via its ability to rescue the sensitivity of yeast cells to camptothecin (a Top1
253 poison), and to also suppress etoposide sensitivity (a Top2 poison) indicating that human
254 TDP2 is active when expressed in *S. cerevisiae* cells (25). Given our findings that TDP2 has
255 the appropriate biochemical activity to remove Spo11 from both ssDNA and dsDNA *in vitro*
256 (above), we were interested to investigate whether TDP2 expression during *S. cerevisiae*
257 meiosis would permit removal of Spo11 *in vivo*.
258

259 To test this idea, we placed His-tagged TDP2 under the control of the *ADH1* promoter in a
260 *sae2*Δ strain, and induced cells to enter meiosis (Fig 4a). Genomic DNA was harvested at
261 hourly intervals during meiotic prophase, and used to probe the DSB hotspot at the
262 *HIS4*::*LEU2* locus as in earlier experiments (Fig 2 and Fig 3). We observed no difference in
263 the abundance or electrophoretic mobility of the DSB bands in either the presence or absence
264 of TDP2 expression, indicating that TDP2 was neither promoting resection nor repair of the
265 Spo11-DSBs (Fig 4a). Expression of TDP2 from an inducible *GAL1* promoter construct after
266 4 hours in meiosis in a *sae2*Δ strain also resulted in no detectable alteration in DSB signal
267 (data not shown).
268
269 A possible explanation for no visible resection or repair of Spo11-DSBs after TDP2
270 expression is that TDP2 has removed Spo11 from the DSB end, but these DSBs, with 2 nt 5′
271 overhangs, are resistant to nucleases such as Exo1 (as demonstrated *in vitro* in Fig 3), and
272 that NHEJ components may be downregulated in meiosis perhaps due to increasing CDK
273 activity as has been observed in cycling *S. pombe* cells (42). To test this possibility, we
274 isolated genomic DNA from a 10 h meiotic time point from cells expressing *TDP2,* or from
275 control cells, and incubated with lambda exonuclease (Fig 4b). At this time point, if TDP2
276 was capable of removing Spo11 from the 5′ end of the DSBs, lambda exonuclease would be
277 able to resect these DSBs (as described in Fig 2). However, we detected no visible resection
278 in the TDP2-expressing nor control cells (Fig 4b). These results suggest that despite the
279 ability of TDP2 to suppress topoisomerase-induced DNA damage in *S. cerevisiae* (25),
280 Spo11-DSBs are refractory to processing by the TDP2 enzyme.
281
282 **Ectopic expression of TDP2 rescues sensitivity of *sae2*Δ and *mre11-H125N* mutant cells**
283 **to etoposide and camptothecin.**
284 As a final investigation of TDP2 activity *in vivo* in *S. cerevisiae* cells, we expressed TDP2 in
285 drug-sensitive (*pdr1*Δ*)* haploid cells (43), and tested for the rescue of camptothecin (CPT)
286 and etoposide sensitivity, as well as to the DNA-alkylating agent methyl methanesulphonate
287 as a control (MMS). At the concentrations tested, wild type control cells (*pdr1*Δ) were not
288 sensitive to etoposide or camptothecin, and there was no effect of TDP2 expression (Fig 4c).
289 *sae2*Δ cells were sensitive to the higher concentration of etoposide and CPT, and were
290 rescued efficiently by ectopic TDP2 expression (Fig 4c). By contrast, whilst loss of Mre11
291 nuclease activity (*mre11-H125N*) resulted in drug sensitivities that were similar to the *sae2*Δ

292 mutant, TDP2 expression caused much weaker suppression than in the *sae2*Δ cells—
293 particularly following CPT treatment (Fig 4c). These observations suggest a separable role
294 for Sae2 and Mre11 in the repair of Top1- and Top2-induced DNA lesions, with TDP2 only
295 able to fully complement the functions of the Sae2 protein. Cells bearing a full deletion of
296 *MRE11* were highly sensitive to even the lower drug concentrations, with no rescue of
297 growth observed upon expression of TDP2 (Fig 4c). This latter result is consistent with the
298 inability for TDP2 to complement some or all of the many functions of Mre11 (44). Notably,
299 TDP2 expression suppressed a very minor growth defect of *sae2*Δ and *mre11-H125N* cells in
300 the presence of MMS (compare colony sizes in Fig 4c with and without TDP2 expression),
301 suggesting either that MMS treatment leads to the accumulation of topoisomerase-lesions, or
302 that it generates directly or indirectly, other potentially toxic intermediates that are substrates
303 for the phosphodiesterase activity of TDP2.
304
305 **DISCUSSION**
306
307 TDP2 was identified in a screen for mammalian genes that rescued the sensitivity of *S.*
308 *cerevisiae* cells to the Top1 poison, camptothecin (25). Biochemical analyses subsequently
309 demonstrated the preferred activity of TDP2 against synthetic 5′ phosphotyrosine groups
310 (25), as well as larger peptides derived from partially proteolysed Top2 (31). Most recently,
311 TDP2 was shown to be proficient in catalysing the release of intact Top2 from dsDNA ends
312 in a reaction that is strongly dependent upon the ZATT protein (45). Here we have
313 characterised the activity of TDP2 against the 5′-linked Spo11 complexes that arise during
314 the induction of meiotic recombination. Recombinant human TDP2 displays robust removal
315 of partially proteolysed Spo11 protein from both ssDNA oligos and dsDNA molecules
316 derived from meiotic cell extracts. We find that non-proteolysed Spo11 is also released albeit
317 with reduced efficiency.
318
319 Under normal conditions in meiosis, Spo11-DSBs are processed by the nuclease activities of
320 Mre11 in conjunction with Sae2, followed by extensive 5′ to 3′ resection mediated by Exo1.
321 Surprisingly, despite efficient *in vitro* removal of Spo11 from DSB ends by TDP2, we have
322 found that the resulting protein-free DNA ends remain very poor substrates for Exo1, in
323 contrast to DSB substrates containing a terminal 3′ single-strand region of ~300 nt. These
324 observations agree with prior observations indicating a preference of Exo1 for partially

single-stranded substrates (12, 41). However, our experiments are the first that employ physiological frequencies of DSB ends relative to genome content (approx. 1 DSB end for every 150 kb), and lead us to conclude that excess dsDNA acts as a competitive inhibitor of Exo1's ability to initiate resection at clean, relatively blunt, dsDNA ends—but not at dsDNA ends that have already undergone limited resection.

Such competitive inhibition may also influence the activity of other proteins involved in DSB end resection. For example, the Sae2-stimulated cleavage of protein-linked dsDNA by Mre11, has thus far only been demonstrated on short, synthetic dsDNA substrates (11–13). Attempts to reconstitute a similar Mre11 endonuclease reaction on natural Spo11-DSBs isolated from meiotic cells has thus far proved unsuccessful in our laboratory (D. Johnson and M. Neale, unpub. obs.)—perhaps in agreement with the reduced efficiency of the Mre11 endonuclease reaction on longer synthetic substrates (Ref 13 and E. Canavo and P. Cejka, unpub. obs.). It is interesting to note that in *S. cerevisiae* Mre11 is loaded at meiotic recombination hotspots prior to Spo11-DSB formation (46). It is tempting to speculate that this interaction is important to ensure efficient nucleolytic processing of Spo11-DSB ends in *S. cerevisiae*—thereby ensuring that meiotic DSB repair is directed down the HR repair pathway.

In *C. elegans*, loss of COM-1 function (the Sae2/CtIP orthologue) leads to aberrant DSB repair and meiotic chromosome segregation due to activation of the NHEJ repair pathway (21, 22). Such observations suggest that mechanisms other than Mre11 are competent to release Spo11 from meiotic DSB ends, generating ligatable DNA ends—at least under conditions when the preferred Mre11-pathway is disrupted. An orthologue of human TDP2 exists in worms (33), but it is yet to be determined whether this activity is the one responsible for enabling NHEJ repair of Spo11-DSBs in the *com-1* mutant.

Interestingly, whilst human TDP2 is capable of catalysing Spo11 removal from DSB ends *in vitro*, we were unable to detect Spo11 removal *in vivo* following ectopic expression of TDP2 within meiotic *S. cerevisiae* cells. Whilst it is hard to rule out a technical reason for observing this negative result, we *were* able to observe robust rescue of camptothecin and etoposide sensitivity in *sae2*Δ and *mre11*-'nuclease-dead' mutant strains upon TDP2 expression. Thus, our favoured interpretation is that Spo11-DSB ends in *S. cerevisiae*—unlike topoisomerase-DNA lesions—are inaccessible to the TDP2 phosphodiesterase activity. Such inaccessibility

may be due to a higher order protein complex present at meiotic DSBs, which are known to require directly or indirectly at least a dozen independent proteins (47). Such ideas are also consistent with observations suggesting multimerisation of the Spo11 enzyme within recombination hotspots (D. Johnson and M. Neale, manuscript in preparation). Alternatively, or in addition, topoisomerase-induced lesions may be subject to greater rates of proteolysis and/or denaturation than Spo11, thereby stimulating the end-cleaning activity of TDP2.

Recognition, and appropriate repair, of DNA lesions is critical for cell survival. During meiotic prophase, the channelling of Spo11-DSBs down the HR pathway has further importance to enable crossover formation, reductional chromosome segregation, and for the generation of genetic diversity (48). Our observations here provide insight into the mechanisms of repairing such protein-linked DSB ends created by topoisomerase-like enzymes such as Spo11. Looking more broadly, our findings may also help to explain some of the genetic differences observed for the resection and repair of ‘clean’-ended site-specific DSBs created by nucleases versus the complex DSB ends created by ionising radiation, which may be a mixture not just of DNA ends with base damage, but also those with blunt or partial 5′ extensions, or occluded by larger protein moieties.

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AUTHOR CONTRIBUTIONS

D.J. and M.J.N. designed and performed the experiments and wrote the paper. E.C. and P.C provided essential reagents and technical supervision.

387 **METHODS**

388

389 **Strains, Plasmids and Recombinant Proteins.** Meiotic experiments utilised the *S.*
390 *cerevisiae* SK1 background (49), and harboured the *sae2*Δ::kanMX or *exo1*Δ::kanMX full
391 gene deletions (9). For Spo11-oligo enrichment, strains harboured the Spo11-
392 $FLAG_3His_6$::kanMX construct (30). The multicopy constitutive P_{ADH1}*TDP2* plasmid (pDJ85)
393 was prepared after replacing the *URA3* gene in pVTU260TT (25) with a Hygromycin
394 resistance cassette. Cell viability spot tests utilized the S288c background harbouring the
395 dominant multi-drug sensitizing cassette *pdr1*Δ::*pdr1DBD-CYC8* (referred to as '*pdr1*Δ')
396 (43) and additionally harbouring the *sae2*Δ::kanMX, *mre11*Δ::*URA3* gene deletions or
397 *mre11-H125N* point mutation (50). Genetic modifications were introduced into yeast strains
398 using standard procedures (51). Recombinant human His-TDP2 was a gift from K. Caldecott,
399 and prepared as described previously (25). Recombinant *S. cerevisiae* Exo1-FLAG and RPA
400 was prepared as described previously (41).

401

402 **Isolation and analysis of meiotic DNA breaks.** SK1 cells were induced synchronously into
403 meiosis using standard procedures (52). At indicated timepoints, 10 ml of cell culture was
404 spheroplasted in 1M sorbitol / 50 mM EDTA / 50 mM $NaHPO_4$ buffer, and lysed by addition
405 of SDS to 0.5% in the presence of proteinase K at 60˚C. Nucleic acids were purified by
406 phenol extraction and ethanol precipitation, and resuspended in 1×TE. DSB signals at the
407 *HIS4*::*LEU2* recombination hotspot were detected using standard techniques (53). Briefly
408 DNA was digested with *Pst*I, electrophoresed on 0.7% agarose in 1×TAE for approximately
409 18 h at room temperature, transferred to nylon membrane under denaturing conditions then
410 hybridized with a probe that recognizes the *MXR2* locus. Prior to digestion, as appropriate,
411 samples were equilibrated in reaction buffer (25 mM HEPES, pH 8.0, 130 mM KCl, 1 mM
412 DTT, 10 mM MgCl2) and incubated with 300 nM recombinant TDP2 for 1 hour at 37˚C, 5
413 units of lambda exonuclease (NEB) for 1 hour at 37˚C, 15 units of Klenow (3′→5′ exo–)
414 polymerase (NEB) with 66 nM dNTPs for 30 minutes at 30˚C, 20 mM recombinant Exo1-
415 FLAG with 680 nM RPA (REF) for 30 minutes at 30˚C, or combinations thereof as indicated
416 in each figure. Reactions involving Exo1 were performed in a similar buffer (25 mM Tris
417 acetate, pH 7.5, 1 mM DTT, 5 mM magnesium acetate, 100 mM sodium acetate, 0.25 mg/mL
418 BSA) in which TDP2 maintains activity (Figure S1b). Reactions were stopped and DNA
419 extracted by the addition of 1 volume of water and 1 volume of phenol:chloroform:isoamyl

420 alcohol (25:24:1) prior to restriction digestion and electrophoresis. Radioactive signals were
421 collected on phosphor screens, scanned with a Fuji FLA5100 and quantified using
422 ImageGauge software.
423
424 **Isolation and analysis of Spo11-oligo complexes.** Spo11-oligonucleotide complexes were
425 detected by immunoprecipitation and end-labelling following established methods (54).
426 Briefly, cells were broken in 10% ice-cold TCA using zirconium beads and a BioSpec 24.
427 Precipitated material was dissolved in SDS buffer, diluted with Triton X100, and Spo11 was
428 immunoprecipitated from total soluble protein using anti-FLAG antibody (Sigma) and
429 protein-G-agarose (Roche). Oligonucleotide complexes were labelled with alpha-^{32}P dCTP
430 using terminal deoxynucleotidyl transferase (Fermentas) and fractionated on a 7.5% SDS-
431 PAGE gel. Alternatively, radiolabelled Spo11-oligo complexes were precipitated with 90%
432 ethanol at –80˚C overnight without or following digestion with trypsin or proteinase K
433 (Fisher) for 1 hour at 37˚C. Precipitated material was collected by centrifugation, solublised
434 in reaction buffer (25 mM HEPES, pH 8.0, 130 mM KCl, 1 mM DTT, 10 mM MgCl2), and
435 incubated with the stated concentration of recombinant TDP2 for 30 minutes at 30˚C prior to
436 denaturation in formamide loading dye and separation on 19% urea/PAGE/1×TBE.
437 Radioactive signals were collected on phosphor screens, scanned with a Fuji FLA5100 and
438 quantified using ImageGauge software.
439
440 **Cell viability spot tests.** YPD cultures were grown in the presence of 300 μg ml^{-1}
441 hygromycin (Invitrogen) to maintain selection of the plasmids. Overnight cultures were
442 diluted 40-fold in fresh YPD containing 300 μg ml^{-1} hygromycin and grown for 4 hours.
443 Cultures were diluted to 0.2 OD_{600} and a 10-fold dilution series prepared before spotting out 3
444 μl onto the YPD plates containing 300 μg ml^{-1} hygromycin and the stated drug dose or
445 DMSO solvent control. Plates were incubated at 30 °C for 3 days prior to image acquisition.
446
447

448 **FIGURE LEGENDS**

449

450 **Figure 1: TDP2 can cleave the covalent phosphotyrosyl bond between Spo11 and a**
451 **single-stranded oligonucleotide.** a-b, Cartoon of the early stages of meiotic recombination.
452 Spo11 generates DSBs and is released covalently attached to short oligonucleotides that are
453 isolated by immunoprecipitation, and end-labelled at the 3′OH using alpha-^{32}P dCTP and
454 TdT, before resolving by SDS-PAGE (b). c-f, Schematic of the experiment (c). Labelled
455 Spo11-oligo complexes without (d) or after treatment with Trypsin (e) or Proteinase K (f),
456 were reacted with recombinant TDP2 at the indicated concentrations, then resolved on 18%
457 polyacrylamide gel under denaturing conditions. Solid arrowheads indicate the migration
458 position of the various Spo11-oligo species (prior to TDP2 treatment). Open triangles and
459 dashed lines indicate the migration position of Spo11-oligos after Spo11-peptide removal by
460 TDP2. Dotted connectors between gels indicate relative migration positions. Asterisks are a
461 non-specific band.

462

463 **Figure 2: TDP2 can remove Spo11 peptides from the dsDNA end of natural Spo11-**
464 **DSBs enabling 5′-3′ resection by lambda exonuclease.**
465 a, Schematic of experiment. b, Genomic DNA isolated from meiotic *sae2*Δ cells was
466 incubated with lambda exonuclease (λexo) without or with prior treatment with TDP2. DNA
467 was purified and digested with *Pst*I, separated by electrophoresis on a 0.7% agarose gel,
468 blotted to nylon membrane and hybridised with a probe (*MXR2* locus) close to the well-
469 characterised meiotic recombination hotspot *HIS4*::*LEU2*. The location of the two major
470 Spo11-DSBs at this locus are marked with solid arrowheads. Disappearance of these signals
471 following TDP2 and lambda exonuclease treatment is due to rapid exonucleolytic
472 degradation from the uncapped Spo11-DSB ends.

473

474 **Figure 3: Recombinant Exo1 is unable to initiate 5′-3′ resection on 3′-recessed and**
475 **blunt-ended Spo11-DSBs even when uncapped by recombinant TDP2, but can**
476 **efficiently resect 5′-recessed Spo11-DSBs that have been processed by Mre11 *in vivo*.**
477 a-b, Genomic DNA isolated from meiotic *sae2*Δ cells was incubated with recombinant Exo1
478 without (a) or with (b) prior treatment with TDP2. c-f, Genomic DNA isolated from meiotic
479 *exo1*Δ cells was incubated without (c), or with recombinant Exo1 (d), TDP2 (e), or TDP2
480 then Exo1 (f). c-d, As in (a, b), but TDP2-uncapped Spo11-DSB ends were blunted by

481 treating with Klenow polymerase + dNTPs prior to incubation with Exo1. DNA was purified
482 and digested with *Pst*I, separated by electrophoresis on a 0.7% agarose gel, blotted to nylon
483 membrane and hybridised with a probe (*MXR2* locus) close to the *HIS4*::*LEU2* meiotic
484 recombination hotspot. The location of the two major Spo11-DSBs at this locus are marked
485 with solid arrowheads. In *exo1*Δ cells, *in vivo* removal of Spo11 from DSB ends and partial
486 resection causes the DSB signals to migrate slightly faster than in *sae2*Δ samples. Further
487 smearing (open triangles) and subsequent disappearance of these signals following Exo1
488 treatment is due to rapid exonucleolytic degradation from the partially resected DSB ends.
489 Lower panels are schematics of each set of reactions.
490
491 **Figure 4: Ectopic expression of *TDP2* cannot remove Spo11 from DSB ends *in vivo*, but**
492 **can suppress camptothecin and etoposide sensitivity.**
493 a-b, Genomic DNA was isolated at the indicated timepoints from a synchronous meiotic
494 culture of *sae2*Δ cells harbouring either an empty vector, or one expressing *TDP2* from the
495 *ADH1* promoter (P_{ADH1}*TDP2*). DNA was purified and digested with *Pst*I, separated by
496 electrophoresis on a 0.7% agarose gel, blotted to nylon membrane and hybridised with a
497 probe (*MXR2* locus) close to the *HIS4*::*LEU2* meiotic recombination hotspot. The location of
498 the two major Spo11-DSBs at this locus are marked with solid arrowheads. In *sae2*Δ cells,
499 Spo11 is not removed and resection cannot occur, causing DSB species to migrate as a tight
500 band. Expression of TDP2 does not alter this migration pattern (a). b, DNA from the 10 h
501 timepoints was incubated with lambda exonuclease for the indicated length of time. c, The
502 indicated strains harbouring either an empty vector, or one expressing *TDP2* from the *ADH1*
503 promoter (P_{ADH1}*TDP2*) were grown to log phase, serial diluted (10-fold), and spotted onto
504 plates containing 300 µg/ml hygromycin (HYG), to maintain plasmid selection, and the
505 indicated concentrations of etoposide, camptothecin (CPT) or methyl methanesulphonate
506 (MMS), and incubated for 3 days at 30˚C.
507
508 **Figure 5: Model of DSB repair via HR or NHEJ depending on repair mechanism.**
509 Covalently bound protein at DNA ends has to be removed in order for repair to occur. Mre11
510 and Sae2 remove the protein block (Spo11 or topoisomerases) nucleolytically, generating a 3′
511 ssDNA overhang substrate that is refractory to NHEJ, but the preferred substrate for long-
512 range resection by Exo1, thereby promoting homologous recombination (left and right
513 panels). Removal of covalently bound protein, for example, by hydrolytically cleaving the

514 phosphotyrosyl bond between the protein and the 5′ end of the DSB (e.g. by TDP2) generates
515 clean, short 5′ overhang ends that are refractory to resection by Exo1—thus potentially
516 inhibiting repair by HR—but the complementary nature of the two ends may promote repair
517 by NHEJ, for example at Top2-DSBs (central panel). The inability for ectopic expression of
518 TDP2 to suppress the requirement for Sae2 in S. *cerevisiae* meiosis suggests that Spo11-
519 DSBs, unlike Topoisomerase-DNA breaks, are inaccessible to TDP2, perhaps due to a large
520 complex at the Spo11-DSB site occluding access to TDP2 (dotted circle in left panel).
521
522 **Figure S1: Recombinant TDP2 is free of nuclease activity, and is active in the preferred**
523 **Exo1 reaction buffer.** a, To test for trace nuclease activity within the recombinant TDP2
524 preparation, a 30 nt oligonucleotide of random sequence was 3′-labelled using TdT and
525 radiolabelled dCTP then incubated with the stated concentrations of TDP2 at 37 °C for 1
526 hour then separated by denaturing urea-PAGE (19% acrylamide). The 30 nt oligo is indicated
527 with a filled arrowhead. **b,** Proteinase K-treated 3′-labelled Spo11-oligo complexes as in Fig
528 1f were incubated with the indicated concentration of TDP2 at 37˚C for 1 hour in Exo1
529 reaction buffer (25 mM Tris acetate, pH 7.5, 1 mM DTT, 5 mM magnesium acetate, 100 mM
530 sodium acetate, 0.25 mg/mL BSA) then separated by denaturing urea-PAGE (19%
531 acrylamide). Solid arrowheads indicate the migration position of Spo11-oligo species (prior
532 to TDP2 treatment). Open triangles indicate the migration position of Spo11-oligos after
533 Spo11-peptide removal by TDP2.

534 **Table 1. *S. cerevisiae* strains used in this study.**

535

Strain	Background	Genotype	Origin
VG296	SK1	*MATa/α, ho::LYS2/", lys2/", ura3/", arg4-nsp/arg4-bgl, leu2::hisG/", his4X::LEU2/" nuc1::LEU2/", SPO11-His6-FLAG3-loxP-KanMX-loxP/"*	This study
VG303	SK1	*MATa/α, ho::LYS2/", lys2/", ura3/", arg4/", leu2/", his4X::LEU2/" nuc1::LEU2/", SPO11-His6-FLAG3-loxP-KanMX-loxP/", sae2Δ::KanMX6/"*	This study
VG164	SK1	*MATa/α, ho::LYS2/", lys2", ura3/", arg4-nsp/", leu2::hisG/", his4X::LEU2/", nuc1::LEU2/", exo1Δ::KanMX4/"*	This study
DJ160	SK1	*MATa/α lys2/", ura3/", arg4/", leu2/", his4X::LEU2/" nuc1::LEU2/", SPO11-His6-FLAG3-loxP-KanMX-loxP/", sae2Δ::KanMX6/" + pDJ85 (P_{ADH1}His6-TDP2)*	This study
DJ199	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2 + pVG19 (empty HYG vector)*	This study
DJ209	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2 + pDJ85 (P_{ADH1}His6-TDP2)*	This study
DJ201	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, sae2Δ::KanMX6 + pVG19 (empty HYG vector)*	This study
DJ193	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, sae2Δ::KanMX6 + pDJ85 (P_{ADH1}His6-TDP2)*	This study
DJ203	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, mre11Δ::KanMX4 + pVG19 (empty HYG vector)*	This study
DJ195	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, mre11Δ::KanMX4 + pDJ85 (P_{ADH1}His6-TDP2)*	This study
DJ212	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, mre11(H125N) + pVG19 (empty HYG vector)*	This study
DJ213	S288c	*MATa, ho, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, pdr1Δ::PDR1-DBD-CYC8::LEU2, mre11(H125N) pDJ85 (P_{ADH1}His6-TDP2)*	This study

536

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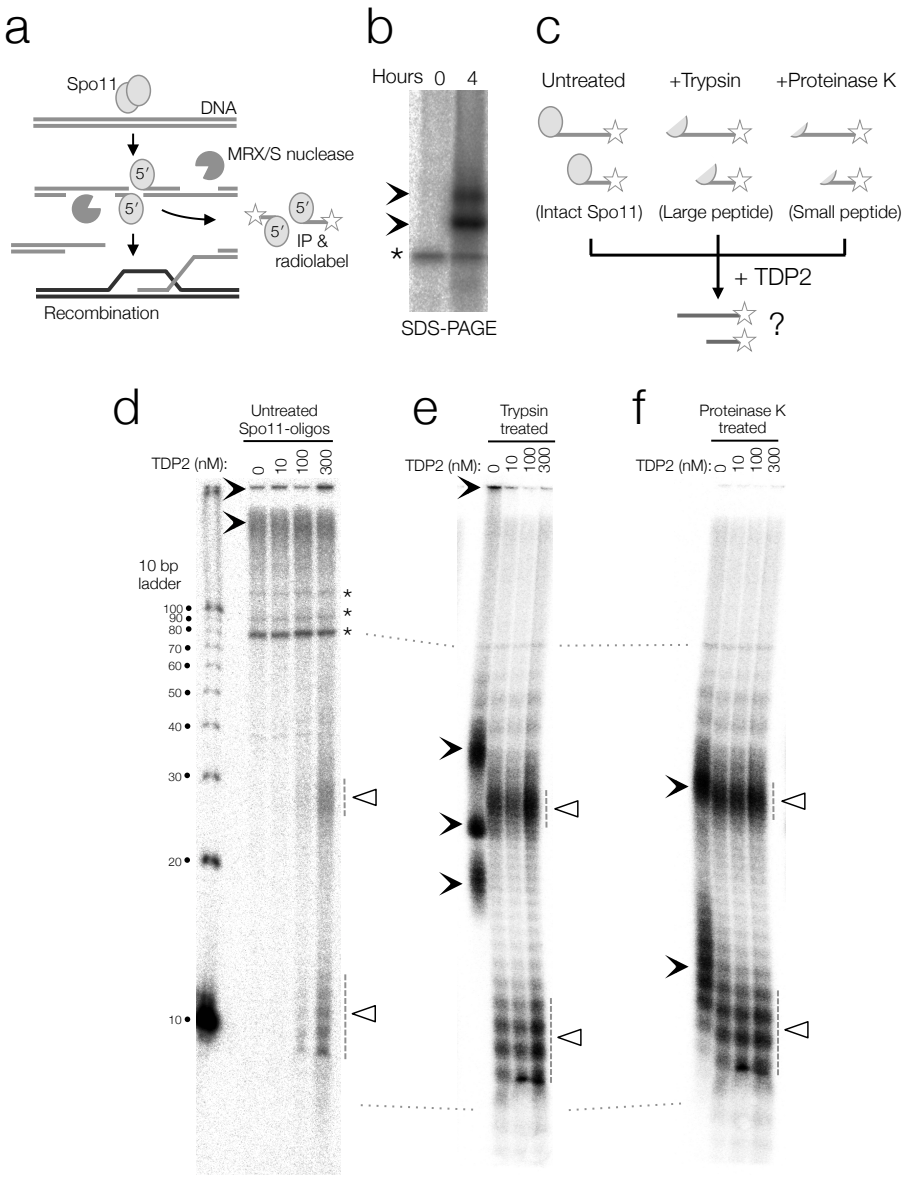

Figure 1: TDP2 can cleave the covalent phosphotyrosyl bond between Spo11 and a single-stranded oligonucleotide. a-b, Cartoon of the early stages of meiotic recombination. Spo11 generates DSBs and is released covalently attached to short oligonucleotides that are isolated by immunoprecipitation, and end-labelled at the 3′OH using alpha-^{32}P dCTP and TdT, before resolving by SDS-PAGE (b). c-f, Schematic of the experiment (c). Labelled Spo11-oligo complexes without (d) or after treatment with Trypsin (e) or Proteinase K (f), were reacted with recombinant TDP2 at the indicated concentrations, then resolved on 18% polyacrylamide gel under denaturing conditions. Solid arrowheads indicate the migration position of the various Spo11-oligo species (prior to TDP2 treatment). Open triangles and dashed lines indicate the migration position of Spo11-oligos after Spo11-peptide removal by TDP2. Dotted connectors between gels indicate relative migration positions. Asterisks are a non-specific band.

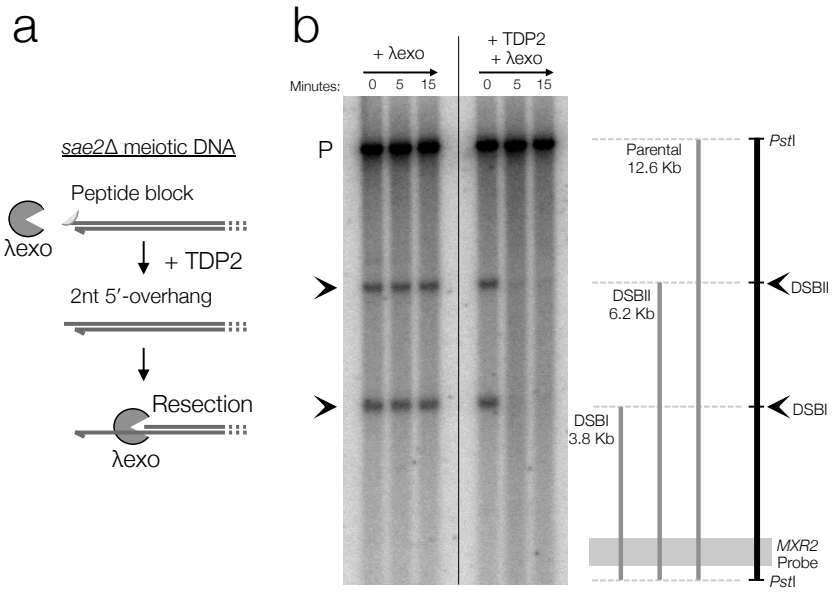

Figure 2: TDP2 can remove Spo11 peptides from the dsDNA end of natural Spo11-DSBs enabling 5′-3′ resection by lambda exonuclease. a, Schematic of experiment. b, Genomic DNA isolated from meiotic *sae2*Δ cells was treated with proteinase K and incubated with lambda exonuclease (λexo) without or with prior treatment with TDP2. DNA was purified and digested with *Pst*I, separated by electrophoresis on a 0.7% agarose gel, blotted to nylon membrane and hybridised with a probe (*MXR2* locus) close to the well-characterised meiotic recombination hotspot *HIS4*::*LEU2*. The location of the two major Spo11-DSBs at this locus are marked with solid arrowheads. Disappearance of these signals following TDP2 and lambda exonuclease treatment is due to rapid exonucleolytic degradation from the uncapped Spo11-DSB ends.

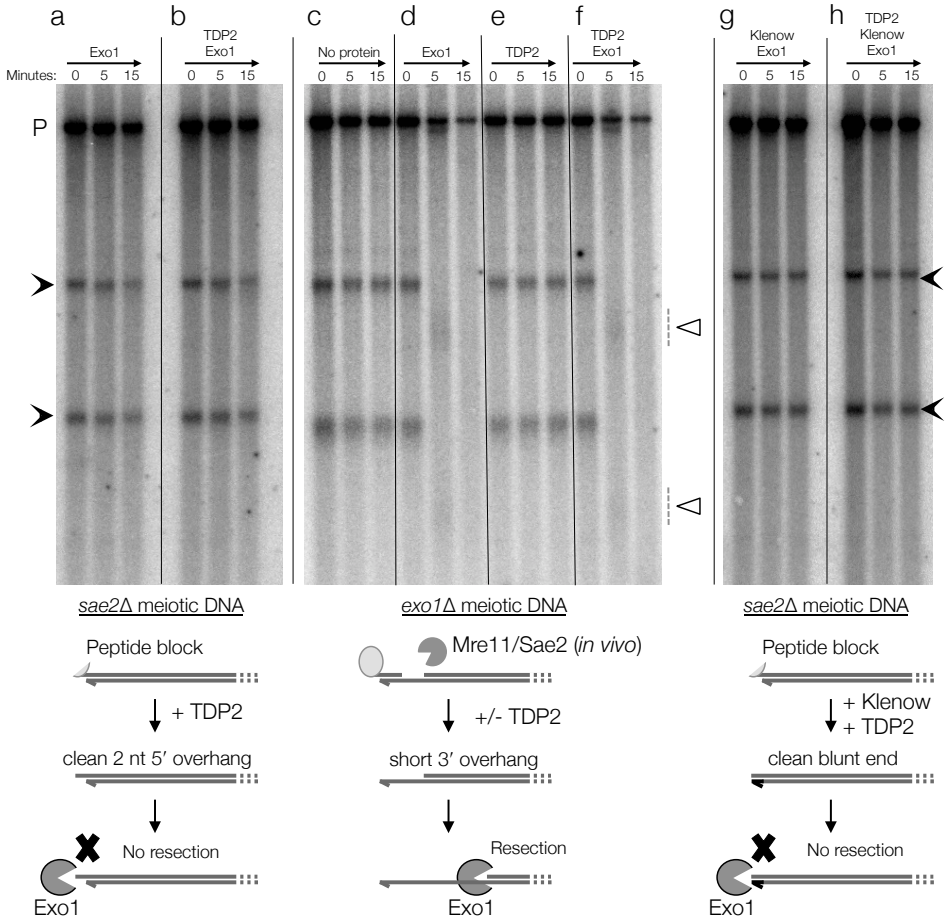

Figure 3: Recombinant Exo1 is unable to initiate 5′-3′ resection on 3′-recessed and blunt-ended Spo11-DSBs even when uncapped by recombinant TDP2, but can efficiently resect 5′-recessed Spo11-DSBs that have been processed by Mre11 *in vivo*.
a-b, Genomic DNA isolated from meiotic *sae2*Δ cells was incubated with recombinant Exo1 without (a) or with (b) prior treatment with TDP2. c-f, Genomic DNA isolated from meiotic *exo1*Δ cells was incubated without (c), or with recombinant Exo1 (d), TDP2 (e), or TDP2 then Exo1 (f). c-d, As in (a, b), but TDP2-uncapped Spo11-DSB ends were blunted by treating with Klenow polymerase + dNTPs prior to incubation with Exo1. DNA was purified and digested with *Pst*I, separated by electrophoresis on a 0.7% agarose gel, blotted to nylon membrane and hybridised with a probe (*MXR2* locus) close to the *HIS4*::*LEU2* meiotic recombination hotspot. The location of the two major Spo11-DSBs at this locus are marked with solid arrowheads. In *exo1*Δ cells, *in vivo* removal of Spo11 from DSB ends and partial resection causes the DSB signals to migrate slightly faster than in *sae2*Δ samples. Further smearing (open triangles) and subsequent disappearance of these signals following Exo1 treatment is due to rapid exonucleolytic degradation from the partially resected DSB ends. Lower panels are schematics of each set of reactions.

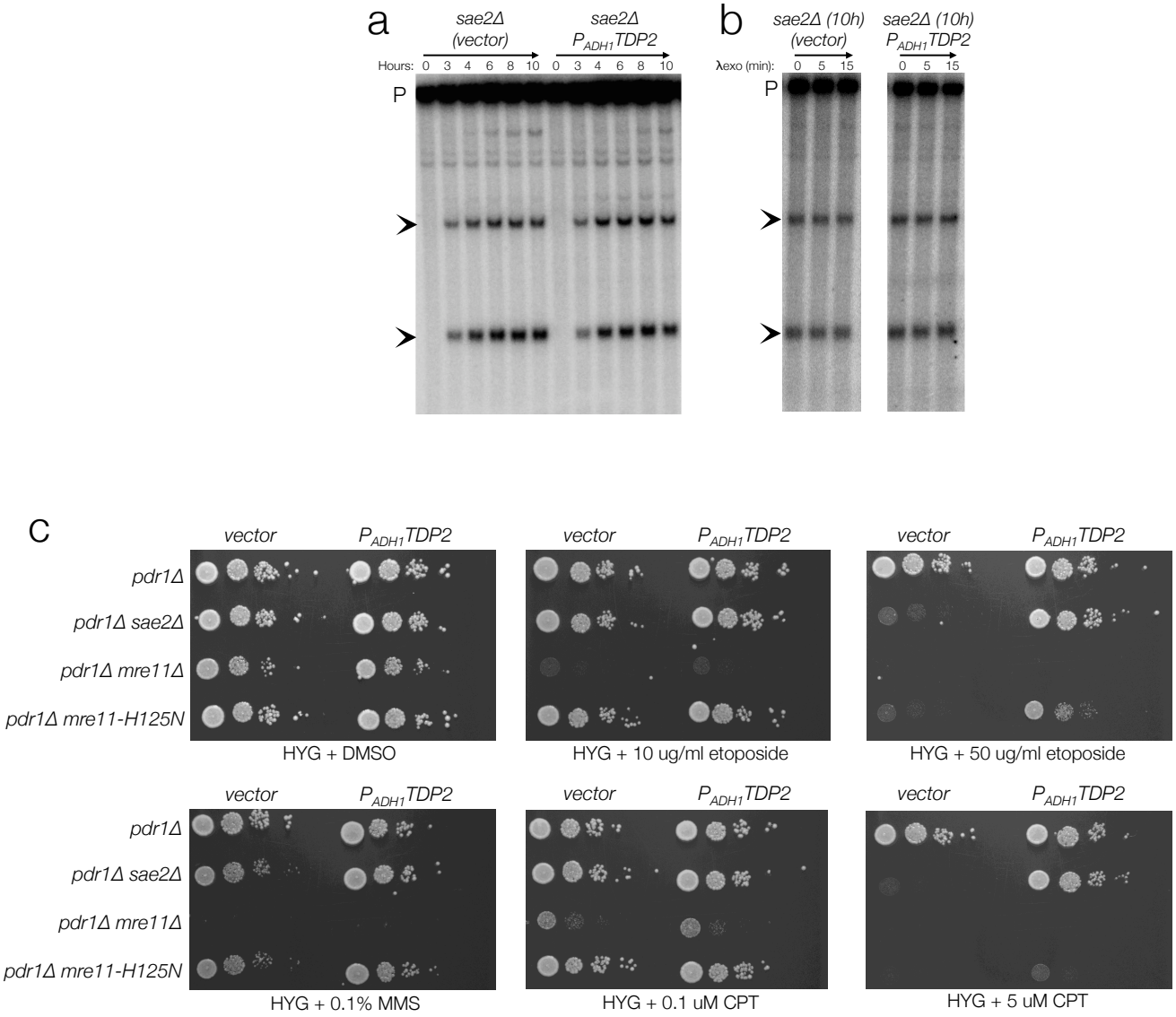

Figure 4: Ectopic expression of *TDP2* cannot remove Spo11 from DSB ends *in vivo*, but can suppress camptothecin and etoposide sensitivity. a-b, Genomic DNA was isolated at the indicated timepoints from a synchronous meiotic culture of *sae2*Δ cells harbouring either an empty vector, or one expressing *TDP2* from the *ADH1* promoter (P_{ADH1}*TDP2*). DNA was purified and digested with *Pst*I, separated by electrophoresis on a 0.7% agarose gel, blotted to nylon membrane and hybridised with a probe (*MXR2* locus) close to the *HIS4*::*LEU2* meiotic recombination hotspot. The location of the two major Spo11-DSBs at this locus are marked with solid arrowheads. In *sae2*Δ cells, Spo11 is not removed and resection cannot occur, causing DSB species to migrate as a tight band. Expression of TDP2 does not alter this migration pattern (a). b, DNA from the 10 h timepoints was incubated with lambda exonuclease for the indicated length of time. c, The indicated strains harbouring either an empty vector, or one expressing *TDP2* from the *ADH1* promoter (P_{ADH1}*TDP2*) were grown to log phase, serial diluted (10-fold), and spotted onto plates containing 300 µg/ml hygromycin (HYG), to maintain plasmid selection, and the indicated concentrations of etoposide, camptothecin (CPT) or methyl methanesulphonate (MMS), and incubated for 3 days at 30˚C.

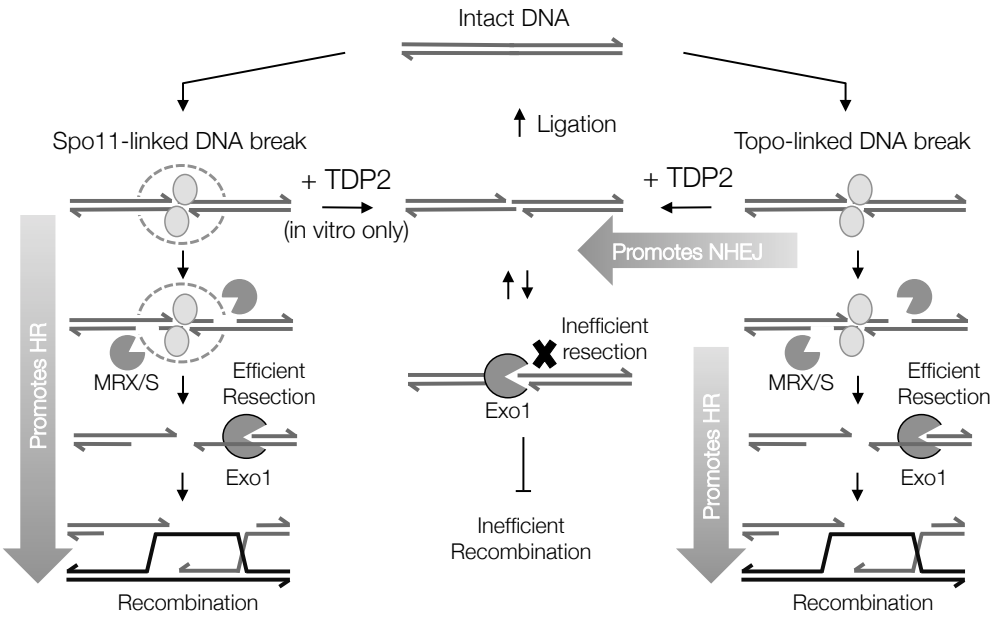

Figure 5: Model of DSB repair via HR or NHEJ depending on repair mechanism. Covalently bound protein at DNA ends has to be removed in order for repair to occur. Mre11 and Sae2 remove the protein block (Spo11 or topoisomerases) nucleolytically, generating a 3′ ssDNA overhang substrate that is refractory to NHEJ, but the preferred substrate for long-range resection by Exo1, thereby promoting homologous recombination (left and right panels). Removal of covalently bound protein, for example, by hydrolytically cleaving the phosphotyrosyl bond between the protein and the 5′ end of the DSB (e.g. by TDP2) generates clean, short 5′ overhang ends that are refractory to resection by Exo1—thus potentially inhibiting repair by HR—but the complementary nature of the two ends may promote repair by NHEJ, for example at Top2-DSBs (central panel). The inability for ectopic expression of TDP2 to suppress the requirement for Sae2 in S. *cerevisiae* meiosis suggests that Spo11-DSBs, unlike Topoisomerase-DNA breaks, are inaccessible to TDP2, perhaps due to a large complex at the Spo11-DSB site occluding access to TDP2 (dotted circle in left panel).

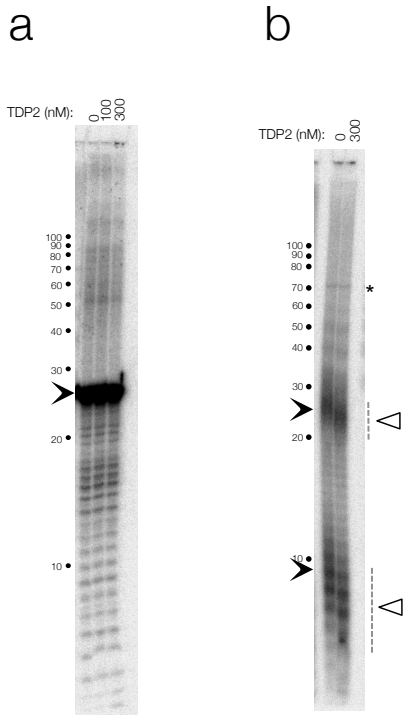

Figure S1: Recombinant TDP2 is free of nuclease activity, and is active in the preferred Exo1 reaction buffer. a, To test for trace nuclease activity within the recombinant TDP2 preparation, a 30 nt oligonucleotide of random sequence was 3′-labelled using TdT and radiolabelled dCTP then incubated with the stated concentrations of TDP2 at 37 °C for 1 hour then separated by denaturing urea-PAGE (19% acrylamide). The 30 nt oligo is indicated with a filled arrowhead. **b,** Proteinase K-treated 3′-labelled Spo11-oligo complexes as in Fig 1f were incubated with the indicated concentration of TDP2 at 37˚C for 1 hour in Exo1 reaction buffer (25 mM Tris acetate, pH 7.5, 1 mM DTT, 5 mM magnesium acetate, 100 mM sodium acetate, 0.25 mg/mL BSA) then separated by denaturing urea-PAGE (19% acrylamide). Solid arrowheads indicate the migration position of Spo11-oligo species (prior to TDP2 treatment). Open triangles indicate the migration position of Spo11-oligos after Spo11-peptide removal by TDP2.